

THE CATALYTIC PROPERTIES OF Fe–S CLUSTER CONTAINING ENZYMES

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St. John's College
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A thesis submitted to the Board of the Faculty of Physical Sciences, for
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Abstract

Many enzymes contain iron– sulfur (Fe–S) clusters which have a huge impact on their catalytic properties. These clusters may form part of the active site or form an electron relay system from the surface of the protein to the active site. Protein film electrochemistry (PFE) was utilized to elucidate the properties of some Fe–S cluster enzymes, namely, Hyd-1(a hydrogenase with an Fe–S electron relay), PceA (a reductive dehalogenase containing Fe–S clusters to facilitate electron transfer with redox partner) and CODH I_{Ch} and CODH II_{Ch} (carbon monoxide dehydrogenases with Fe–S electron relay systems and Ni– incorporated Fe–S clusters as active sites). The role of a proline residue at the active site in Hyd-1 was investigated and it was concluded that some local instability and adverse effect on H_2 activation were introduced upon replacement of proline with an alanine residue. The PceA dehalogenase was studied with PFE in terms of their interactions with various substrates and inhibitors. Furthermore, a method for performing ‘film correction’ for liquid substrates as that of the dehalogenase was established. Aspects of the catalytic cycle and effects of oxygen (O_2), peroxide (H_2O_2) and hydroxylamine (NH_2OH), a nitrogen– containing peroxide analogue on CODH I_{Ch} and CODH II_{Ch} were investigated with PFE. Finally, Electrochemical Impedance Spectroscopy (EIS), a technique involving application of alternating current (AC), was added to the portfolio was PFE techniques to compare CpI and $CrHydA1$ (hydrogenases with and without Fe–S electron relay system, respectively) in terms of time-dependent and time-independent processes within them. A novel term, *exchange catalytic rate*, for expressing inherent proficiency of the enzyme at zero-current potential was proposed and quantified. A means for measuring electroactive coverage and theoretical turnover *during* catalysis in PFE experiments was developed.

Collaborations

Protein samples have been generously provided by the research groups of Prof. S. Ragsdale (CODH I_{Ch} and CODH II_{Ch}: University of Michigan, USA), Prof. Thomas Happe (*CpI* and *CrHydA1*: Ruhr-Universität Bochum, Germany) and Prof. Gabriele Diekert (*PceA*: Friedrich-Schiller- Universität Jena, Germany). The NMR experiments for detection and quantification of formate were carried out in collaboration with Dr. Nick Rees (CRL, University of Oxford, UK). Ammonia detection and quantification were performed by Dr. Ian McPherson (Tsang group, ICL, University of Oxford, UK). Experiments for detection and calibration of tetrachloroethylene (PCE), trichloroethylene (TCE) and *cis*-dichloroethylene (*cis*-DCE) by GC-MS technique were carried out by Dr. James Wickens (CRL, University of Oxford, UK) while the Differential Scanning Calorimetry (DSC) experiments for measuring the protein unfolding temperatures of native and variant Hyd-1 were performed by Dr. David Staunton (Dept. of Biochemistry, University of Oxford, UK). Preparations of native and variant Hyd-1 for crystallography were made by Elena Nomerotskaia (lab technician, FAA Group, ICL, University of Oxford) while the crystals were formed and modelled by Dr Stephen Carr (Research Complex at Harwell (RCaH), Didcot, UK). The Hyd-1 work is part of a long-term collaboration effort among past and present FAA group members Dr. Rhiannon Evans, Sara Wehlin, Gerri Roberts and Emily Brookes. Impedance techniques were carried in collaboration with Dr. Kavita Pandey (then visiting student to FAA group, ICL, University of Oxford, UK). The CODH – O₂/hydride works were carried out with Vincent Wang (then senior DPhil student from FAA group, ICL, University of Oxford, UK).

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Publications

Articles published during my DPhil programme (based on works presented in this thesis) are given below:

1. Kavita Pandey, **Shams T. A. Islam**, Thomas Happe, Fraser A. Armstrong
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‘Importance of the Active Site "Canopy" Residues in an O₂-Tolerant [NiFe]-Hydrogenase’
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3. B. Siritanaratkul, **Shams T. A. Islam**, T. Schubert, C. Kunze, T. Goris, G. Diekert and Fraser A. Armstrong
‘Selective, light-driven enzymatic dehalogenations of organic molecules’
RSC Adv., **2016**, *6*, pp 84882–84886
4. Vincent C. -C. Wang, **Shams T.A. Islam**, Mehmet Can, S. W Ragsdale, F. A. Armstrong
‘Investigations by Protein Film Electrochemistry of Alternative Reactions of Nickel-Containing Carbon Monoxide Dehydrogenase’
J. Phys. Chem. B, **2015**, *119* (43), pp 13690–13697

List of Abbreviations and Symbols

A	area of the electrode/ pre-exponential factor of Arrhenius equation
A	ampere, unit of current
AP	artificial photosynthesis
BV	Butler-Volmer
CA	chronoamperometry
C_d/C_{dl}	capacitance of the double layer
C_e	capacitance of the enzyme
c_g	concentration of dissolved gas
CODH I _{Ch}	carbon monoxide dehydrogenase I from <i>Carboxydotherrnus hydrogenoformans</i>
CODH II _{Ch}	carbon monoxide dehydrogenase II from <i>Carboxydotherrnus hydrogenoformans</i>
CPE	constant phase element
CpI	Hydrogenase-1 from <i>Clostridium pasteurianum</i>
CrHydA1	Hydrogenase A1 from <i>Chlamydomonas reinhardtii</i>
CV	cyclic voltammetry
Cp	specific heat under constant pressure
DBE	dibromoethylene
DC	direct current
DCE	dichloroethylene
DCP	dichlorophenol
DFT	density functional theory
d_o	distance above closest approach of the electron donor and acceptor
ds	double strand
DSC	differential scanning calorimetry
DMF	dimethyl formamide
E	applied potential
$E^{\circ'}$	formal redox potential
E_a	activation energy
<i>E. coli</i>	<i>Escherichia coli</i>
EIS	electrochemical impedance spectroscopy
ENDOR	electron nuclear double resonance
$E_{Ox/R}$	reduction potential of the electrochemical control centre
EPR	electron paramagnetic resonance
E_{switch}	potential at which two potential-dependent states interconvert
ET	electron transfer
eV	electron volt
F	Faraday constant = 96,485 Cmol ⁻¹
f	constant equivalent to F/RT
Fe _d	distal iron
Fe _p	proximal iron
Fe-S	iron-sulfur

FLP	frustrated lewis pair
FTIR	fourier transform infrared spectroscopy
h	Planck constant = 6.626×10^{-34} Js
H_{AB}^2	squared electronic coupling element
HyaA	small subunit of Hyd-1
HyaB	large subunit of Hyd-1
HyaC	b-type cytochrome, redox partner of Hyd-1
Hyd-1	Hydrogenase-1 from <i>E. coli</i>
$\tilde{I}$	induced current in AC systems
$i/i_{obs}/I$	observed current
i_{cat}	current due to catalysis
i_e	current across the resistance offered at the interface of electrode
i_f	faradaic current
i_{max}	current at 100% Substrate
i_{nf}	non-faradaic current
i_o	exchange current density/exchange catalytic rate
I_o	amplitude of current (I) in AC systems
IsC	iron sulfur cluster biosynthesis pathway
i_{trans}	current across the resistance of solution due to mass transfer limitations
j	complex number of $\sqrt{(-1)}$ flux of electrons to and fro from the electrode surface
k	rate constant
K_M	Michaelis constant
k_B	Boltzmann constant= 1.381×10^{-23} JK ⁻¹
k_{cat}	catalytic rate
k_{ex}	Henry's law constant
k^o/k_{ex}	exchange rate constant
kDa	kiloDalton
MES	2-(N-morpholino)ethanesulfonic acid
MQ	Milli-Q
MWNT	multiwalled nanotube
n	the number of electrons
Nif	nitrogen fixation biosynthesis pathway
NMR	nuclear magnetic resonance
OD	optical density
PCE	tetrachloroethylene
PceA	tetrachloroethylene reductive dehalogenase
PCR	polymerase chain reaction
PDB	protein data bank
PFE	protein film electrochemistry
P_g	partial pressure of gas

PGE	pyrolytic graphite electrode
Py	1-pyrenebutyric acid
QM/MM	quantum mechanics/molecular mechanics
Q^n	pre-factor of CPE (constant phase element)
R	Universal Gas Constant = 8.314 J / mol. K Resistance in DC system
R_e	resistance offered at equilibrium potential in impedance measurements
rpm	revolutions per minute
R_s	solution resistance
Rr	<i>Rhodospirillum rubrum</i>
S	substrate
SCE	standard calomel electrode
SDS	sodium dodecyl sulfate
SHE	standard hydrogen electrode
Suf	sulfur formation biosynthesis pathway
t	time
T	absolute temperature
TCE	trichloroethylene
T_m	protein unfolding temperature
ν	scan rate
V	applied voltage in DC systems
$\tilde{V}$	applied voltage in AC systems
V_o	amplitude of voltage in AC systems
WT	wildtype
Z	impedance of AC systems
Z_o	amplitude of impedance in AC systems
Z_w	impedance of Warburg element
α	transfer coefficient
$\emptyset$	phase shift between voltage and current in AC systems
$^{\circ}\text{C}$	degree Celsius
Å	angstrom
β	medium-dependent decay constant
βd_o	effective tunnelling factor
Γ	electroactive coverage
ΔG^o	standard Gibb's free energy change
$\Delta H^{\ddagger}$	enthalpy change
$\Delta S^{\ddagger}$	change in entropy
η	overpotential
λ	reorganization energy
σ	Warburg coefficient
ω	perturbation frequency
W	Warburg element

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Chapter 1: Introduction

1.1 Fe–S clusters: Occurrence, Types and Role in Electron Transfers

Iron-sulfur (Fe–S) clusters are the most ubiquitous of cofactors¹ – inorganic groups bound to the protein scaffold of enzymes– and are found in both prokaryotes and eukaryotes. Since their discovery in the 1960s, more than 100 Fe–S clusters have been uncovered of which more than 50% are reported to be involved in electron transfer while a further 17% are involved in non-redox catalysis.² In addition, they are also involved in regulation of gene expression and enzyme activity, in iron storage and in donation of sulfur atoms.³

The Fe–S clusters are usually bound to the protein scaffold by cysteine residues though other ligands such as histidine, arginine, aspartate and serine or back-bone amides have been reported.⁴ The type and number of residues involved seems to have a role in tuning the redox potential of the cluster, for example, for electron transfers, Fe–S clusters can access an array of conformations whose reduction potentials may range from –600 mV to 400 mV.⁵ These clusters impart various properties to the protein in which they are incorporated: for redox catalysis, Fe–S clusters with very low redox potentials can carry out reduction reactions; for non-redox catalysis, the Fe–S clusters can allow small compounds to bind to them as they have Lewis acid properties;² and for the ability to regulate gene expression, the of Fe–S clusters can reversibly interconvert between their various forms make them ideal in detecting redox– or iron– related stresses.^{2, 6}

Whatever the function and whatever the organism under study, the biosynthesis pathway for Fe–S clusters is one of the following three: the *Isc*(iron sulfur cluster), *Suf*

(sulfur formation) or the *Nif* (nitrogen fixation) biosynthesis pathway.⁷ The *Isc* machinery is for Fe–S clusters meant for housekeeping purposes, the *Suf* machinery is for Fe–S clusters dealing with stress situations and the *Nif* machinery is for specialized Fe–S cluster formations, *e.g.*, formation of those which occur in nitrogenases.⁸

Specialized Fe–S clusters are those that are not the normal rhombic [2Fe–2S] or cubic [3Fe–4S]/[4Fe–4S] clusters and are normally varied by having larger polynuclear centres or by being attached to moieties such as a di-iron centre (as occurs in [Fe–Fe] hydrogenases) or by having Cu or Mo or Ni atoms incorporated into them (as in various carbon monoxide dehydrogenases).² In *E. coli*, the cubane [4Fe–4S] clusters account for ~ 90% of the clusters while the [3Fe–4S] and [2Fe–2S] together accounts approximately for the remaining 10%.² Some examples of ‘specialized’ and ‘normal’ [Fe–S] clusters are given in Figure 1.

To understand the role played by Fe–S clusters in electron transfers (ET), it is pertinent to know that these clusters are usually found in nature located within 14 Å of each other in an electron relay systems. Pure ET between two centres placed at certain distances from each other occurs either by tunnelling⁹ (i.e. through space) or via electron hopping¹⁰ (i.e. through bonded orbitals). Tunnelling occurs in essentially quantum mechanical systems whereby there is always a non-zero probability of electrons being allowed to traverse between two points. It may occur even if available energy does not exceed the barrier in between.

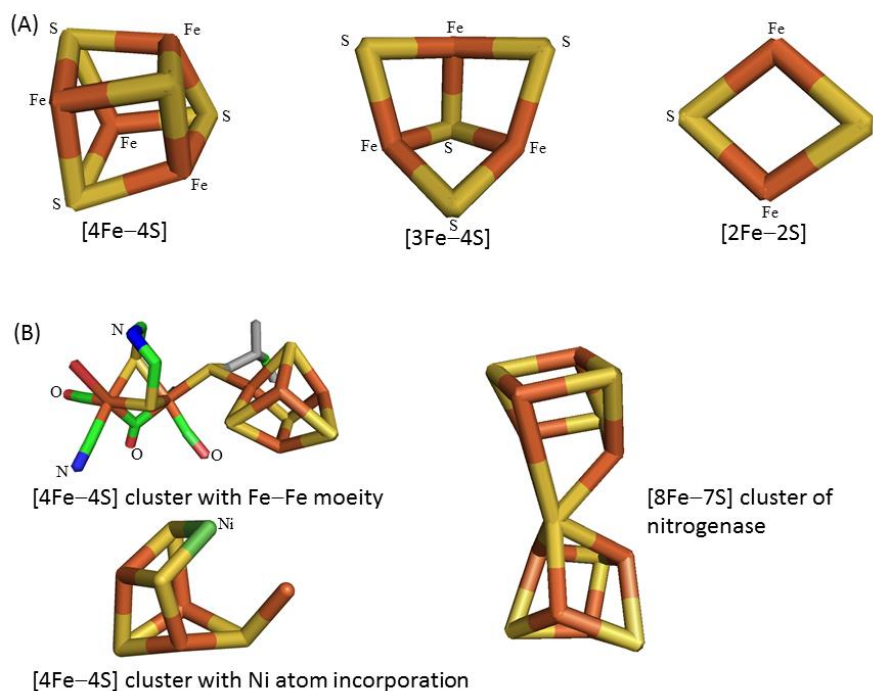


Figure 1: Various Fe-S clusters in nature. (A) The typical Fe-S clusters: cubane [4Fe-4S] and [3Fe-4S] clusters and the rhombic [2Fe-2S] cluster. (B) Examples of specialized Fe-S clusters: from Cpl of *Clostridium pasteurianum* (PDB code: 3C8Y)¹¹ an example of cluster with Fe-Fe moiety, from CODH II_{Ch} of *Carboxydothermus hydrogenoformans* (PDB code: 1SU7)¹² a cluster incorporated with Ni and from nitrogenase MoFe-protein of *Azotobacter vinelandii* (PDB code: 4WNA)⁸ an example of a large polynuclear Fe-S cluster.

On the other hand, hopping is an ET mechanism that will only occur when there is an overlap between the electron donor and electron acceptor states, *i.e.*, for a transient moment of time, the jumping electron is able to exist on the electron donor and acceptor systems simultaneously. Hopping allows electron to pass from donor to acceptor to another acceptor so that long-range ET becomes possible as shown in Figure 2.

Electron transfer rates in outer-sphere reactions (macroscopic systems) and inner-sphere reactions (microscopic systems) were elucidated by Rudolph A. Marcus and won him a Nobel Prize in 1992 for his work.¹³ He gave the absolute rate of ET as:¹⁴

$$k_{ET} = \left(\frac{4\pi^3}{\lambda k_B T h^2} \right)^{\frac{1}{2}} H_{AB}^2 \exp(\Delta G^\ddagger) \quad \text{Equation 1}$$

by invoking $\Delta G^\ddagger = -\frac{(\lambda + \Delta G^0)^2}{4\lambda RT}$ where, λ is the reorganization energy, *i.e.*, the energy needed to bring the nuclei from^{9, 15} the equilibrium position of the reactants to that of the products and has to be provided by ΔG^0 , the change in standard Gibb's free energy of the system.

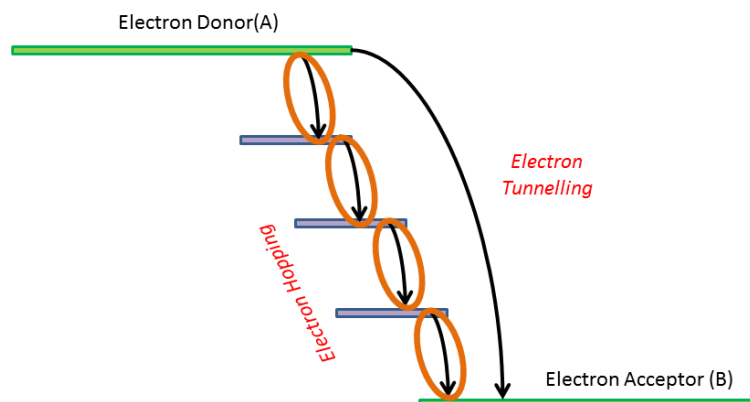


Figure 2: Electron tunnelling vs Electron hopping. Electron traversing from the electron donor (A) to the acceptor (B) states (green bars) are shown as occurring through two mechanisms. In electron tunnelling, the transfer occurs in one-step and is dependent on the difference between the standard Gibbs's free energies of the acceptor and donor states (ΔG^0) and on the reorganization energy (λ) involved in forming the acceptor state from the donor state. Electron hopping occurs as a series of electron transfers which form a cascade *via* bond formation among the elements involved (blue bars).

H_{AB}^2 is the electronic coupling element squared which equals the probability that an electron will tunnel through the potential barrier that exists between A (electron donor) and B (electron acceptor) and is a constant between them when distance is fixed. The H_{AB}^2 element is distance- dependent with an exponential fall (modified with coefficient β known as the electronic decay factor) in its value upon increased values of distance.^{9, 15-16} The coefficient is usually assigned a constant value of $1.4 \text{ \AA}^{-1}$ and given we deal with a narrow range of 12-14 $\text{\AA}$ distances among electron-transferring elements in nature, the term H_{AB}^2 can be considered a constant. The other constants terms that appear are π (pi), R (universal gas constant) and T (absolute temperature): thus, Equation

implies that for a given system, ΔG^0 and λ are the only variables which impact the rate of ET at a particular temperature.

Since ' $-\Delta G^0$ ' is the driving force arising from the difference in the reduction potentials of donor and acceptor,¹⁷ the rate of ET within an enzyme would depend on the protein environment to which the pair is exposed. Moser-Dutton equation suggests for a system where $\Delta G^0 = 0$ (redox potentials of donor and acceptor systems are matched) and the distance between the donor and acceptor are within 12–14 Å, electron tunnelling would be favoured over electron hopping.^{15b} When tunnelling occurs, the protein matrix essentially acts as an insulating layer between the two centres and the intervening residues do not serve as intermediate states in the ET chain.

The role of the protein matrix is manifested in its influence over ΔG^0 and further in its ability to shield from H₂O (when forming hydrophobic pockets) and oxidation by O₂ (when forming suitable gas channels). These additional abilities ensure that reorganization energies are low for reduction or oxidation.¹⁸ Furthermore, the rigid structures of Fe–S clusters (with notable exception being the proximal [Fe–S] cluster in hydrogenase-1 from *E. coli*¹⁹) and electron's non-entry into anti-bonding orbital facilitate stability of the length of the Fe–S bond and maintain against additional requirement of reorganization energy should electron tunnelling occur. Thus, structures of Fe–S clusters and the protein environments in which they are found are determining factors in the rates at which electron tunnelling occurs. It is reported that distances of 12-14 Å spacing between electron donor and acceptor sites is conducive to electron tunnelling occurring sufficiently fast so as not to affect the rate of catalysis.^{15A}

Seemingly contrary to the observation that a natural ‘within 14Å’ distance between the Fe–S clusters are sufficient to achieve fast enough rates, Fe–S clusters are also found in nature arranged systematically according to a potential gradient^{15b, 20} and bound to the protein framework with ligands that are conserved to maintain their potential.²¹ This contradiction can however be deemed of small import by considering an interesting instance in hydrogenase-1 from *E. coli* whose [3Fe–4S] cluster, located medially in an electron relay system containing three [Fe–S] clusters, has a reduction potential significantly more positive than the clusters adjacent to it on either side.^{22,23} This leads to a tendency for electrons to reside on it. However electron flow to and from the active site *via* this relay system occurs rapidly and reversibly. A similar observation that electron flow remains rapid and reversible, even on ‘uphill’ steps in terms of potential, was also made by Hudson *et al*²⁴ and modelling studies have revealed that the natural ‘within 14Å’ distance does indeed help to overcome these ‘uphill’ steps.^{15b} Thus, it may be worth inferring that the systemic arrangement of Fe–S clusters according to potential and the conserved ligands have evolved for reasons other than to facilitate ET.

The potential(s) of Fe–S cluster(s) near the surface of enzymes determine the potential at which the electrons are able to enter the enzyme: *in vivo*, the electrons come usually from a redox partner, conveniently located within tunnelling distance.²⁵ In some cases, *e.g.*, in the functioning of *E. coli* fumarate reductase respiratory chain, the potentials of these surface Fe–S clusters in alternate enzymes along the respiratory chain determine which of them receive the electrons from the electron shuttling system²⁶, *i.e.*, the electron pathway of the system. Additionally, the difference in the potential of the Fe–S cluster serving as the gateway into/out of the enzyme with that of the reaction occurring at the active site determine the *bias* displayed by the enzyme.²⁷ Like all catalysts,

enzymes are capable of increasing the rates of chemical reactions in *both* directions of a reversible reaction. However, it is evident that most enzymes preferentially increase the rate of reaction in one direction in what is known as the ‘*bias*’ of the enzyme.’²⁸ This *bias* has recently been modelled in terms of the difference in potential of the Fe–S cluster with that of the reaction at the active site by Hexter *et al.*²⁹ The roles of the Fe–S clusters in electron transfer pathway in a respiratory chain and inherent *bias* of the enzyme are visualized in Figure 3.

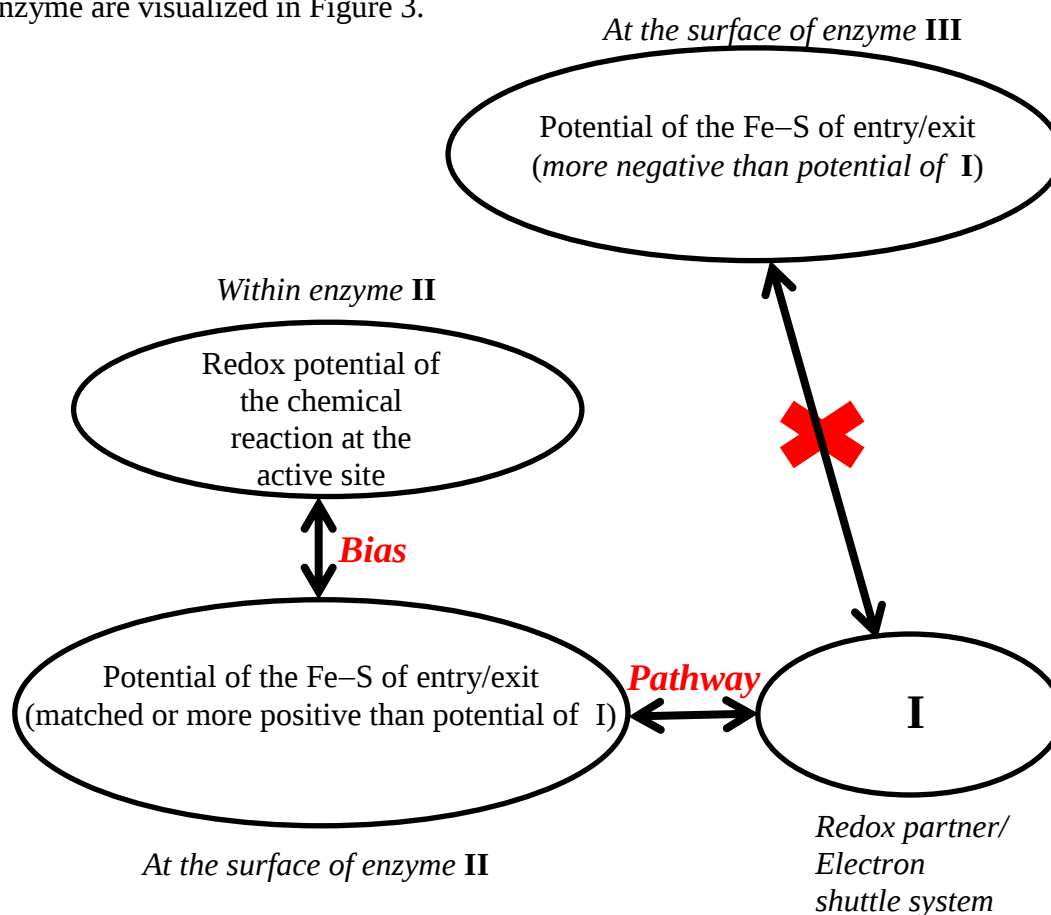


Figure 3: Roles of Fe–S clusters in bias and pathway of electron transfer. Structure **I** represents the redox partner or electron shuttle system from which enzyme **II** and **III** may receive electron. Since enzyme **III** contains a gateway Fe–S cluster whose potential is more negative than that of Structure **I**, electron traverses to enzyme **II** whose entry/exit Fe–S cluster is at a potential more positive or matching that of Structure **I**. Within the enzyme, the difference between redox potential of the chemical reaction at the active site and that of the gateway Fe–S cluster determines the bias of the enzyme. The greater the difference, the greater is the bias as modelled by Hexter *et al.*²⁹ The bias is the characteristic preference that the enzyme displays in increasing the rate of one reaction over that of the reverse reaction.

1.2 Protein Film Electrochemistry (PFE)

Investigations using Protein Film Electrochemistry (PFE) have played an important role in the characterization of various enzymes including hydrogenases and carbon monoxide dehydrogenases over the last two decades.^{28, 30} The technique typically utilizes the application of direct current (DC) voltages to elucidate the mechanisms of enzymes as they undertake catalysis by examination of cyclic voltammograms (CV) and chronoamperometry (CA) results.³⁰

Various enzymes were demonstrated to be excellent *electrocatalysts*, catalysts which enhance electrochemical reactions,^{29,31} by the PFE technique and many of them operate with minimal overpotentials on pyrolytic graphite edge (PGE) electrodes.^{29, 32} In this technique, a minute quantity (generally, 1–2 μl) of the enzyme solution (typically, of 1–10 μM concentration) is applied to an electrode surface to create a thin film which is then mounted as a working electrode in a three-electrode cell set-up. Working electrodes are usually rotated at high rates to eliminate mass transfer limitations while predetermined potentials or a range of potentials (usually -1 V to 0 V vs SCE, Standard Calomel Electrode) are applied *via* a potentiostat. The enzymes are then thoroughly examined for their responses (recorded as currents, usually in the micro-Ampere (μA) range) to elucidate the enzyme activity and catalytic pathway undertaken in terms of the applied potential. The recorded current is proportional to the number of electrons consumed/released during the reaction at the active site of the enzyme provided no other factor is limiting³³ – the electrons either flow from or to the electrode surface which thus serves either as a *source* or a *sink* of these electrons. The current recorded during catalysis, i_{cat} , is related to the turnover of the enzyme, k_{cat} , by the relation:^{22, 30}

$$i_{cat} = A\Gamma F n k_{cat} \quad \text{Equation 2}$$

where A = area of the electrode surface, Γ = electroactive coverage by enzyme, F = Faraday's constant and n = number of electrons involved in the reaction. The exact coverage, Γ , of the enzyme on the electrode usually remains unknown during catalysis, making it hard to calculate enzyme turnover from current values. A technique for determining Γ by PFE is available only during non-turnover conditions (Appendix 1).

Protein film electrochemistry provides an ideal means of studying inhibitor and substrate interactions with immobilized enzymes, especially those which are membrane-bound *in vivo*,^{27, 31-32} and also helps to distinguish between rapid steady-state catalytic activities at different potentials from relatively slow, potential-dependent changes in activity.^{28, 34} Temperature, pH and concentration of substrates and/or inhibitors have influence on the magnitude of the recorded current and by fixing all the variables except one, the import of that variable may be studied. It may be noted that PFE is usually not utilized as a stand-alone approach – complementary information are provided, for example, by spectroscopy studies which define different states of enzymes and kinetic studies which quantify rates under defined experimental conditions. Protein film electrochemistry provides the *relative change* that occurs upon changing a variable as opposed to the absolute change in the rates.

A general approach employed, over the years, for characterization of an electrochemical cell or any solid state device is the one involving the application of a small perturbing potential across it. This method is called Electrochemical Impedance Spectroscopy (EIS) and is an alternating current (AC) technique which has been in use since late 19th century with a report in 1952 of it being used to investigate polarization across biological cell membranes.³⁵ It leads to the modelling of an equivalent electrical circuit

made up of resistors, capacitors and inductors in order to mimic the current-voltage behaviour displayed by the electrochemical cell or sample being studied.³⁵ It may then be inferred which of the components or biophysical processes of the cell or sample have the ‘resistor-like’, ‘capacitor-like’ or ‘inductor-like’ characteristics thus allowing one to gain more knowledge about the system. Decisions then may be taken on how the system is best suited to the purpose or on which modifications need to be undertaken to better suit the purpose.

The use of EIS has proved largely successful in analyzing immunosensor platforms^{36(a-d)} in which immobilized receptive biomolecules on electrodes (typically antibodies, nucleic acids or peptides) convert the target binding event into a measurable electrical signal. It has been possible to elicit reliable data with femto molar concentrations— only a few molecules of pathological protein detection levels have been reported.^{37(a-b)} There are no records of EIS being used for electrodes upon which enzyme has been immobilized to understand and quantify the ‘resistor-like’, ‘capacitor-like’ or ‘inductor-like’ characteristics of enzymes. Components with ‘capacitor-like’ or ‘inductor-like’ characteristics manifest resistances which are mathematically complex in nature, *i.e.*, they have real and imaginary portions. Practically, it means that the current varies with time as the applied voltages also vary with time and the resistance offered by the system is called ‘impedance’.³⁵ This impedance, thus, has real and imaginary components : the real portion does *not* vary with time (resistor-like; time-independent) while the imaginary portion does (‘capacitor-like’ or ‘inductor-like’; time-dependent).

1.3 Introduction to Fe–S containing Enzymes

Enzymes occur ubiquitously in nature and every living organism, from smallest bacteria *Mycoplasma genitalium*³⁸ to plants to humans to whales, produce them to maintain life. Even viruses, of which there is considerable debate as to whether they constitute living things, encode for at least one type of enzyme which enables the copying of their genetic material to that of the host.³⁹ In 1995, Dr. Richard Wolfenden reported of a chemical reaction he considered ‘absolutely essential’ in creating the building blocks of DNA and RNA and that would take 78 million years without enzymes – an apt example that illuminates the importance of enzymes in maintaining life.⁴⁰

Enzymes are ideal examples of catalysts since they increase the rates of reaction without being changed chemically in any way. They are classified into six categories based on the type of reaction they catalyse.⁴¹ Of these, oxidoreductases enzymes, which increase the rates of redox reactions, are the most varied with 22 subclasses depending on the functional group they act upon. In redox reactions, electrons are transferred to the site of reduction and withdrawn from the site of oxidation; the catalysis of these reactions by enzymes usually occurs at the active sites of these enzymes which are regions that bind substances during reactions. This binding helps that substance to undergo the reaction.⁴² In enzymes, active sites are usually found deeply buried within protein frameworks.⁴³ Transfer of electrons to these deeply buried structures are usually facilitated by a Fe–S cluster or by a Fe–S cluster-containing electron relay system. The active sites may also be composed of Fe–S clusters or be modified Fe–S cluster systems.

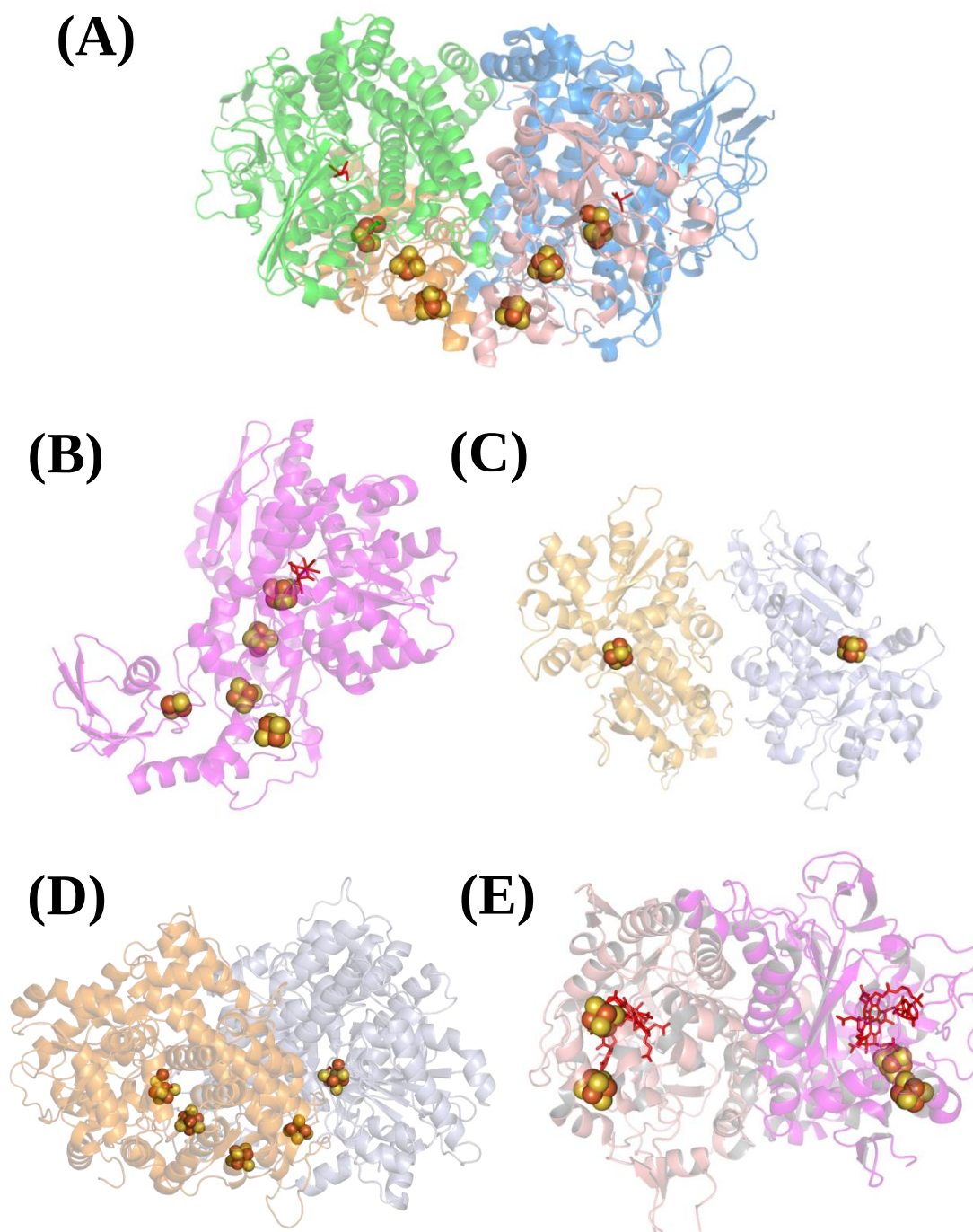


Figure 4: Structure of the enzymes studied in this thesis. (A) Hyd-1 from *E. coli* (PDB code: 5A4M)⁴⁴, (B) Cp1 from *Clostridium pasteurianum* (PDB code: 3C8Y)¹¹, (C) CrHydA1 from *Chlamydomonas reinhardtii*, shown without the bimetallic centre of the active site known as the H-cluster (PDB code: 4R0V)⁴⁵ as no structure of the enzyme have been solved yet with the H-cluster (D) CODH II_{Ch} from *Carboxydotherrmus hydrogenoformans* (PDB code: 1SU7)¹² and (E) PceA from *Sulfurospirillum multivorans* (PDB code: 4UQU)⁴⁶. The Fe-S clusters, as part of electron relays or active sites, are shown as coloured spheres: Fe (orange) and S (yellow). The active site (other than the Fe-S cluster portion) is shown as red coloured sticks.

It is being proposed to study the catalytic properties of some [Fe–S] cluster containing enzymes in the work presented in this thesis. A number of hydrogenase enzymes were utilized, namely, the [Ni–Fe] hydrogenase-1 from *Escherichia coli* (Hyd-1) and [Fe–Fe]-hydrogenases, hydrogenase A1 from the green alga *Chlamydomonas reinhardtii* (CrHydA1) and hydrogenase-1 from the fermentative anaerobe *Clostridium pasteurianum* (CpI). The enzymes carbon monoxide dehydrogenase-I and II from *Carboxydotherrmus hydrogenoformans* (CODH I_{Ch} and CODH II_{Ch}) and a reductive dehalogenase from *Sulfurospirillum multivorans* (PceA) have also been studied. All enzymes are redox active and are unified by the possession of Fe–S clusters as part of their electron relay systems and/or part of their active sites as shown in Figure 4. The details of the enzymes are given below (Sections 1.4 to 1.6) and PFE shall be used for the study along with some complementary techniques. For the first time, the AC technique, EIS, shall be included, among the suite of dynamic electrochemical techniques that is grouped under PFE.

1.4 Hydrogenases

1.4.1 Hydrogen (H₂) as a fuel

A lot of research is being carried out to prepare for an ‘energy crisis’ as fossil fuel supplies dwindle. According to the latest reports, 86.6% of total worldwide energy consumption in 2015 was derived from the combustion of fossil fuels⁴⁷ inspite of detrimental environmental impacts arising from the use of fossil fuels. One avenue of research involves envisioning an economy based on use of hydrogen as fuel. Hydrogen (H₂) is extremely suited as an energy carrier and the product of H₂ combustion is water, making the process green and carbon neutral. Hydrogen can serve as fuel in fuel cell devices which currently uses platinum (Pt) as electrocatalysts. However, Platinum

is not only expensive and finite, but is often poisoned by contaminants of the steam reforming process that supply the H_2 fuel: hydrogen sulphide (H_2S) and carbon monoxide (CO).⁴⁸ Thus, for a H_2 economy, research has to be two-pronged to meet the current and ever-increasing energy demand: production of H_2 must be cleaner and costs must be lowered.

Fuel-making devices such as those envisioned for use in Artificial Photosynthesis (AP) processes may provide the necessary clean H_2 . The concept is to couple highly active enzymes to devices that generate photon-excited electrons from the sun to generate H_2 as fuel, mimicking what occurs naturally in plant leaves/algae, and add to the current value of 5% of overall hydrogen production that come from renewable sources (the rest is mainly derived from fossil fuels).⁴⁹ To bring costs down, widely abundant and low-cost nickel (Ni) and iron (Fe) electrodes can be used in place of Pt in fuel cells, though so far, their use has proven to be inefficient as they require higher temperatures and voltages of 2 volts or higher to be applied.⁵⁰ Although H_2 releases a lot of energy to the point of causing explosion (142 kJ/g- about three times greater than that of gasoline), H_2 is inert to combustion without a catalyst being present to lower its high dissociation enthalpy (436 kJ/ mol). An alternative to using inorganic metals such as Ni and Fe as electrocatalysts is the use of enzymes or biomimetics inspired by them to overcome the high activation energy through formation of a stable enzyme-substrate complex. Enzymes or biomimetics inspired by them are also likely to be able to operate at low temperatures and be impervious to the effects of contaminants such as H_2S and CO in H_2 stock feed.

Enzymes in many microbes are known to catalyse the interconversion between H_2 and H^+ (Equation 3) and as a group form a key aspect of microbial energy cycling in nature.⁵¹ These enzymes are known as hydrogenases and are composed of low cost and abundant metals (Ni and Fe) as part of their active sites and as such can be adequate source of inspiration for biomimetics.^{52,28, 53} Furthermore, hydrogenases have been reported to carry out oxidation of molecular H_2 at rates and efficiencies comparable to those of Pt but in conditions of ambient temperature and pressure (a turnover of 1500 s^{-1} at $30\text{ }^\circ\text{C}$ and pH 7 has been reported).^{54, 30}



1.4.2 Classification of [Ni–Fe] hydrogenases

Convergent evolution has led to the formation of three classes of hydrogenases based on the composition of their active sites: [Ni–Fe], [Fe–Fe] and [Fe] hydrogenases.⁵⁰ In the following sections, the structure of the active sites and the catalytic cycles of [Ni–Fe] and [Fe–Fe] hydrogenases are discussed: [Fe] hydrogenases are not part of this study.

The [Ni–Fe] enzymes are further subdivided into five classes.⁵⁵ Class I, the membrane-bound H_2 -uptake enzymes, include Hyd-1 from *Escherichia coli* (*E. coli*) which facilitates oxidation of H_2 coupled with quinone reduction. The *E. coli* genome is known to encode for three [Ni–Fe] isoenzymes, Hyd-1, Hyd-2 and Hyd-3, whose expressions are determined by environmental conditions and allow the same substrate (H_2) to be used under different conditions. In some cases, these enzymes have sacrificed reversibility and show preference for either H_2 production or H_2 reduction. Hyd-1 is one such enzyme and, at neutral pH *in vitro*, it is a H_2 oxidizer which is also O_2 -tolerant.^{34a} It has a relatively small $K_M^{H_2} = 9\text{ }\mu\text{M}$ (at pH 6.0, -0.11 V)⁵⁶ indicating a high affinity for

its substrate. Hyd-1 has been isolated from *E. coli* via a genetically engineered hexa-his tag which binds to Ni– affinity purification column.^{30, 57} Since tolerance towards O₂ inhibition is a crucial aspect for any technological application, Hyd-1 or any of the biomimetics inspired by it has huge potential in terms of commercial application. Turnover rates 257.4 s⁻¹ for native/wild-type (WT) Hyd-1 from *E. coli* have recently been reported by Evans *et al.*⁴⁴

Class II enzymes are also H₂-uptake enzymes but are found in the cytoplasm and include cyanobacterial uptake hydrogenases as well as H₂-sensing hydrogenases which regulate gene expression according to the H₂ levels they sense. Class III enzymes, also present in the cytoplasm, are bidirectional *in vivo*, *e.g.* nicotinamide adenine dinucleotide phosphate (NADP)–coupled hydrogenase mediate the exchange of reducing equivalents between H₂ and the soluble redox cofactor, NADP. Class IV enzymes are membrane-associated H₂-evolving hydrogenases or energy-converting hydrogenases *e.g.* Hyd-3 from *E. coli*. Class V enzymes are similar to Class I enzymes but is put into a separate class as they lack membrane-targeting signal peptides and share low overall sequence identity.^{54c}

1.4.3 Structure of the active site of [Ni–Fe] hydrogenases

Crystal structures of Hydrogenase-1 (Hyd-1) from *Escherichia coli*, a [Ni–Fe] hydrogenase, were solved recently^{44, 58} and one of them is shown in Figure 5 highlighting its dimeric structure. Hyd-1 is thought to be a heterodimeric protein with each dimeric unit containing a large subunit and a small subunit. The large subunit, HyaB, contains the [Ni–Fe] cofactor while the small subunit, HyaA, harbours the three Fe–S clusters which form a relay from the active site to the surface. The three Fe–S clusters in the

small subunit are named according to their positions relative to the active site with the closest being labelled proximal and the ones further along being labelled as medial and distal clusters, respectively. The proximal cluster and its ability to furnish electrons to reduce O₂ to water molecules is thought to be responsible for the O₂-tolerance of Hyd-1.^{28-29, 34a} The proximal cluster is located 10.6 Å away from the [Ni-Fe] cofactor while the medial cluster is 9.4 Å from the proximal and the distal is situated 8.6 Å from the medial as shown in the zoomed Section (I) of Figure 5.

The [Ni-Fe] cofactor in the large subunit is the site of H₂ activation and catalysis. This bimetallic active site consists of Ni and Fe atoms with the Ni atom being coordinated by four cysteine-S ligands as shown in the zoomed Section (II) of Figure 5. Two of these bridge the Ni to the Fe atom. The Fe atom is then further coordinated to three biologically unusual diatomic ligands, one CO and two CN groups.

The protein scaffold provides well-placed acidic and basic groups to facilitate catalysis.⁴⁴ The bulky protein scaffolds of hydrogenases, in general, also protect the enzymes' cofactors from exposure to water and O₂; recent work has shown that embedded organometallic complexes based upon active sites of hydrogenases can be protected within 3D conductive structures serving as mimics of the scaffold.⁵⁹ The enzyme contains a hydrophobic channel which allow gas molecules to reach the active site (as found by Xe trapping crystallography in [Ni-Fe] hydrogenase of *Desulfovibrio fructosovorans* and Kr trapping in that from *Ralstonia Eutropha*).⁶⁰ There exists, at the small subunit, a hydrophobic transmembrane α -helix that serves to anchor the protein to the membrane. A b-type cytochrome, termed as HyaC, serves as physiological *in vivo* redox partner at which quinone reduction occurs.^{30, 61}

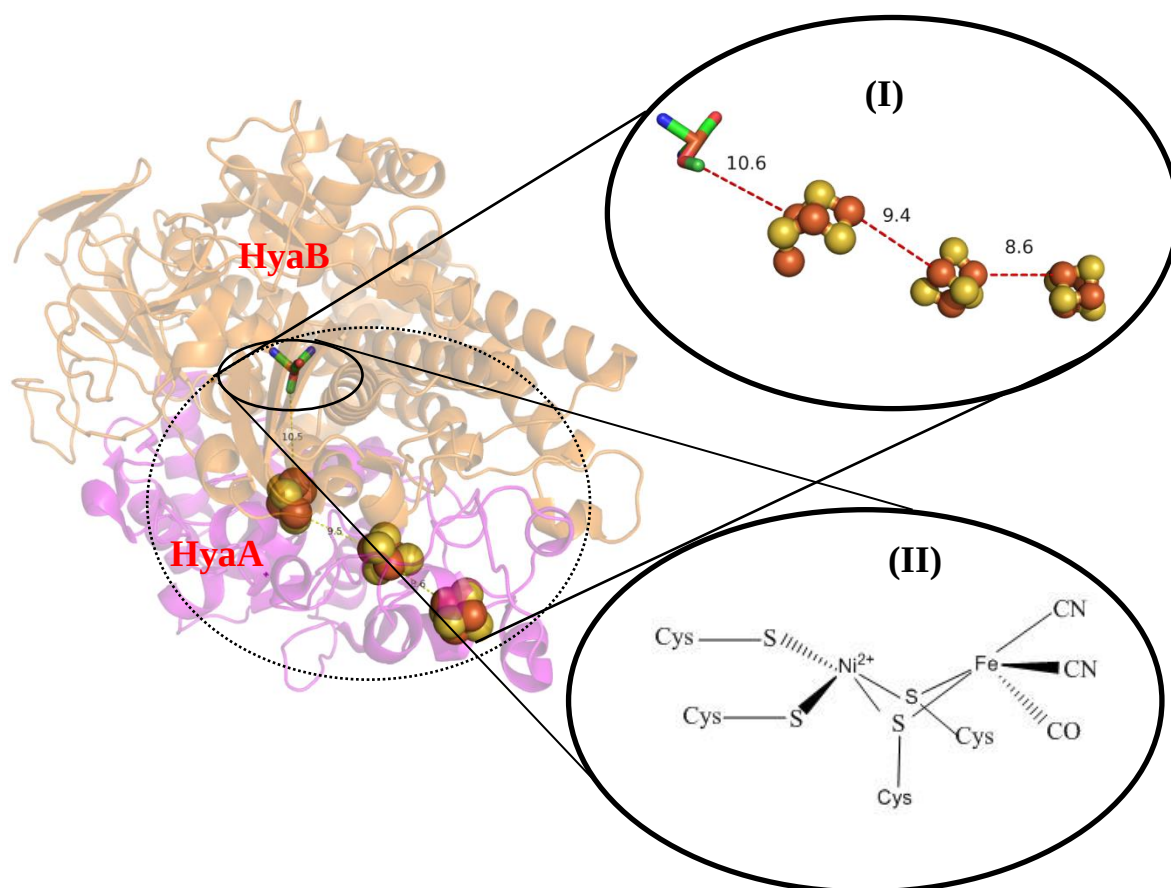


Figure 5: Structure of hydrogenase from *Escherichia coli* highlighting (PDB code: 5A4M)⁴⁴ the dimeric structure. The larger subunit (HyaA) and the smaller subunit (HyaB) are shown. The smaller subunit houses the Fe–S cluster relay system and the zoomed out Section (I) shows the inter-distances between the components in Ångström. The larger subunit houses the site for H₂ activation shown as sticks which is further highlighted in zoomed out Section (II) in a Chem Draw representation.

1.4.4 Catalytic cycle of [Ni–Fe] hydrogenase

One of the proposals dealing with the catalytic cycle of [Ni–Fe] was made by Siegbahn *et al*⁶² and it is considered here. The binding of H₂ for oxidation is proposed to occur at an oxidation state of the active site known as Ni-SI_a in which both Ni and Fe atoms are in the +2 state.⁶² In fact, the Fe atom has been shown to remain in the low-spin Fe⁺² state by Mossbauer, Electron Paramagnetic Resonance (EPR) and Electron Nuclear

Double Resonance (ENDOR) spectroscopy in all of the observable states⁶³ most probably due to being bound to ligands which have π -acceptor characteristics.

In the most oxidised inactive state of the active site of the enzyme, an unpaired electron on the Ni is EPR-visible as an $S = 1/2$ species⁶⁴ and redox titration of it brings about an one-electron reduction to the Ni-SI_a state. Further titration reveals the Ni-R state which is two electrons more reduced than Ni-SI_a. Both the Ni-SI_a and Ni-R states are thought to be involved in the catalytic cycle. A Ni-C state, thought to be a transitional state between these two states, was detected by EPR⁶⁵ and is one-electron more reduced than Ni-SI_a. It is believed to have a hydride ligand bridging the Ni³⁺ and Fe²⁺ atoms.⁶⁴

Density Functional Theory (DFT) suggests that H₂ may be able to bind to both metal atoms of the active site⁶⁶ though the hydrophobic gas channel ends at the Ni atom.⁶⁷ Furthermore, CO, a competitive inhibitor to H₂ oxidation, binds at the Ni site indicating that H₂ binding may occur initially at the Ni atom. However, contrarily, the Fe atom with its vacant binding site and low spin electron configuration, is ideal for initial binding of H₂. Whichever is the binding site, it is known that the binding of H₂ occurs to the Ni-SI_a state of the active site as shown in Figure 6.

After H₂ binding, the next step is most likely the heterolytic cleavage of the H₂ resulting in a hydride which bridges the Ni and the Fe atoms (Ni-R state) from which a loss of electron (e) to the [Fe-S] cluster and a proton yield the Ni-C state. The hydride is then lost as a proton resulting in gain of electrons to reduce Ni³⁺ to Ni¹⁺ and subsequent loss of e to the nearby [Fe-S] and a proton regenerates the Ni-SI_a state.

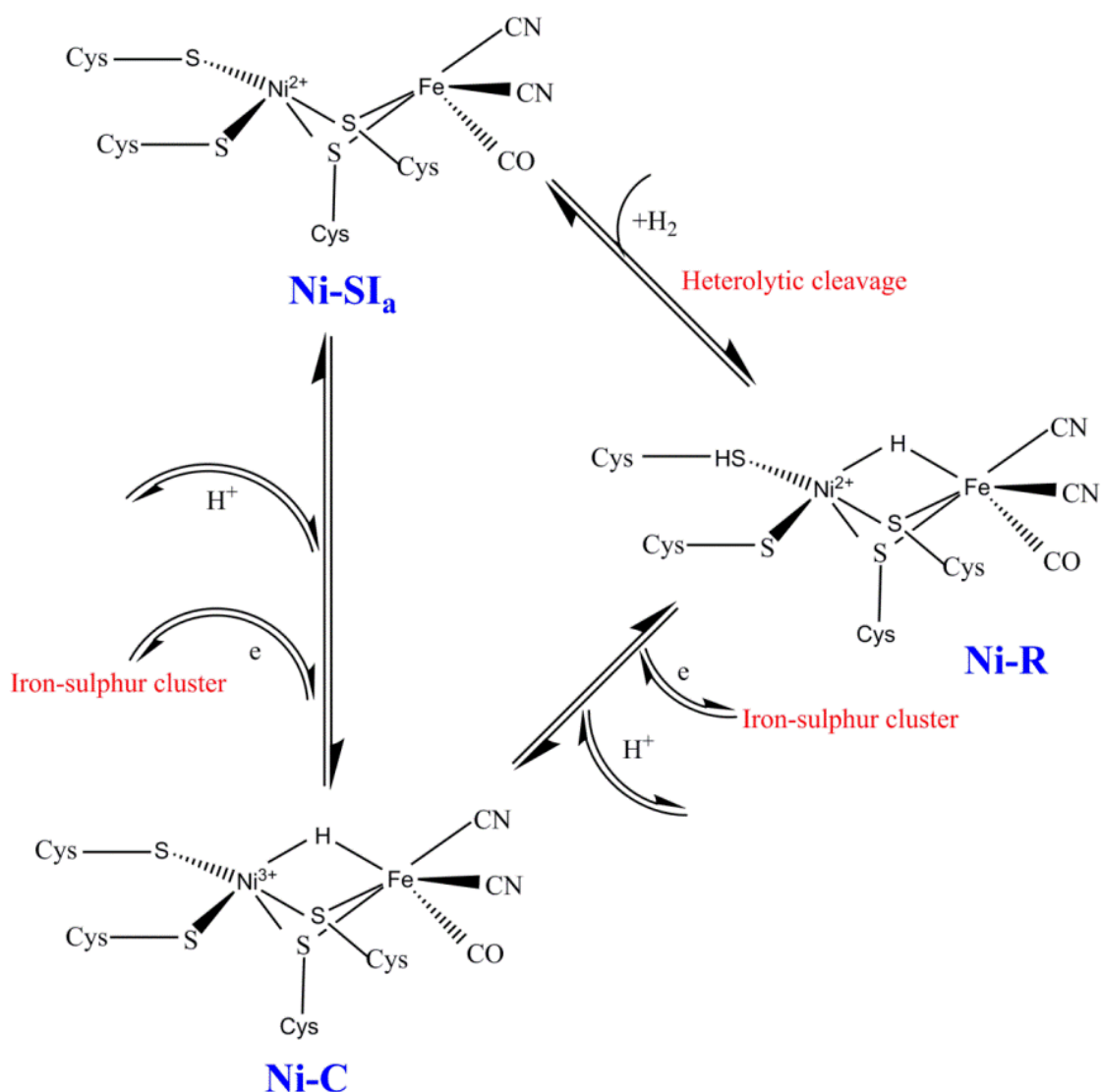


Figure 6: Catalytic cycle of [Ni-Fe] hydrogenase. The proposed cycle by Siegbahn and Hall⁶² show the active site in the Ni-SI_a state being ‘attacked by H₂’ and then cycling through the Ni-R and Ni-C states to recover the Ni-SI_a state by release of 2 protons and 2 electrons. The proposed cycle is supported by DFT and QM/MM studies.

A long-held assumption that [Ni-Fe] hydrogenase uses a thiolate S ligand as the base for heterolytic H-H bond cleavage is shown in Figure 6.^{54a} However, the fact that simple Ni complexes with terminal thiolates are not good catalysts has led to an alternate proposal by Evans *et al* based on studies of Hyd-1 of *E. coli*.⁴⁴ The proposal is that a strictly conserved arginine situated <4.5 Å from the bimetallic active centres (R509; Hyd-1 numbering) serve as the required base. The proposal envisions that activation

involves splitting the H_2 molecule with either the Ni or Fe atom acting as Lewis acid while the nitrogen on the guanidinium group on R509 acts as the base and completes a Frustrated Lewis Pair (FLP) system.⁶⁸ In the reverse direction and in strong acidic environment, the hydrogen atom attached to Ni or Fe atom is expected to be strongly hydridic and therefore basic in character. This hydrido ligand then deprotonates the guanidinium group resulting in the formation of H_2 .

1.4.5 Structure of the active site of [Fe–Fe] hydrogenases

The [Fe–Fe] hydrogenases are found in both bacteria and eukarya⁶⁹ and tend to display a catalytic *bias* towards H_2 production. They are reported to have the highest turnover frequencies (in either directions of H_2/H^+ interconversions) of the three classes of hydrogenases.³²

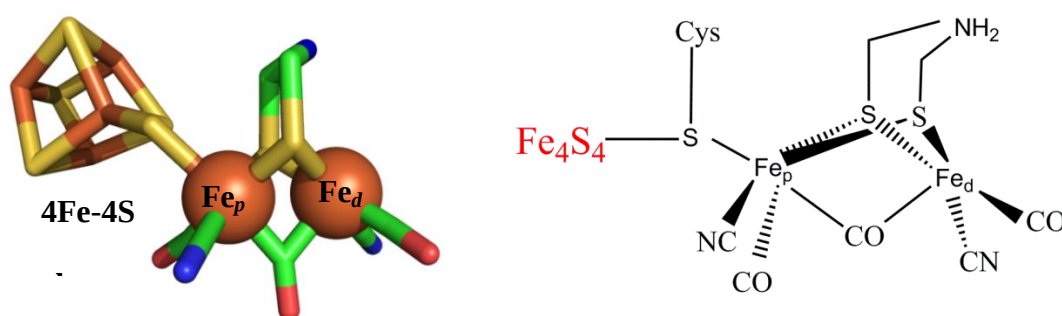


Figure 7: Pymol and Chem Draw representation of the H-cluster of [Fe–Fe] hydrogenases. The Pymol structure is of a [Fe–Fe] hydrogenase, *Cp1*, from *Clostridium pasteurianum* (PDB code: 3C8Y)¹¹ and shows the active site known as the H-cluster composed of a unique di-iron active site (where H_2 binds and is activated) and attached to a [4Fe–4S] cluster which serves both as a conduit and store for electrons. Fe_d and Fe_p refers to the distal and proximal iron atoms based on their position with respect to the [4Fe–4S] cluster. The blue and red sticks in the Pymol structure represent nitrogen and carbon atoms, respectively.

The [Fe–Fe] hydrogenases contain unique bimetallic active sites known as the H-clusters which contains six Fe atoms spread over two domains: a 2Fe domain that is

the site for H₂ activation and a [4Fe–4S] cluster domain that acts as an electron store and conduit.^{54a, 70} The [4Fe–4S] cubane is coupled to the binuclear Fe cluster *via* a S of a nearby cysteine residue as shown in Figure 7. The di-iron entity is incorporated into the protein framework by two cysteines of a nearby α -helix strand. Each Fe atom of the bimetallic centre is coordinated to a terminal CO and a terminal CN ligand, as well as to a dithiolate bridge.¹¹ There is a further CO ligand which is found either bridging both Fe atoms or terminal to the Fe_d (in Figure 7, the bridging configuration is shown). Both CO and CN are uncommon ligands and allow adoption of low oxidation states which allow the readily available transition element to behave similarly to Pt for processing of H₂.

On comparing the amino acid sequences of various [Fe-Fe]-hydrogenases, similarities are found not only in the bimetallic portion of the active site, but are also found in the second coordination sphere involving twelve amino acids.⁷¹ The largest difference between them seem to arise from the number of Fe–S clusters they contain as part of the electron relay system(s).⁷² For example, *Hydrogenase-I* obtained from fermentative anaerobe *Clostridium pasteurianum* (Cp1) contains an electron-transfer relay consisting of three [4Fe–4S] clusters and a [2Fe–2S] cluster in addition to the H-cluster. Another [Fe–Fe] hydrogenase, obtained from photosynthetic algae *Chlamydomonas reinhardtii* (CrHydA1), is known as one of the simplest characterized and contains the H-cluster but no additional relay clusters.

The Fe–S centres of CpI that are adjacent to the H-cluster and part of the electron relay system are detectable by EPR and have an average reduction potential of approximately –420 mV vs SHE (at pH 8).⁷³ Specific values for the two distal Fe–S centres near the surface are currently unknown. The electron relay system allows long-range electron

transport between the H-cluster to the protein surface and it may be significant that it branches into two pathways that could provide improved coupling to a redox partner which, *in vivo*, is a 2[4Fe–4S] ferredoxin.^{73b} Within the living cell, the H-cluster of CrHydA1 receives electrons from Photosystem I *via* a small [2Fe–2S] ferredoxin known as PetF.^{11, 74}

1.4.6 Catalytic cycle of [Fe–Fe] hydrogenase

The three catalytically active sites have been identified as H_{ox} , H_{red} and H_{sred} and one proposal of the catalytic cycle involving them (proposed by Adamska *et al*⁷⁵) is shown in Figure 8. It may be noted that the [4Fe–4S] centre in the H-cluster plays a significant role in the catalytic cycle.

The most oxidized catalytically active state of the enzyme, H_{ox} , is known to catalyse H_2 oxidation in which the Fe_d is in the +2 oxidation state. The Fe_d atom is especially susceptible to attack by exogenous CO: the H_{ox} –CO crystal structure has been solved.⁷⁶ Since CO is a competitive inhibitor of H_2 , it may be assumed that the Fe_d is also the site where H_2 (the substrate) binds and is shown as such in Figure 8. Heterolytic cleavage then occurs such that pendent nitrogen at the bridgehead abstracts a proton and the Fe_d contains a hydride, H^- . It is unclear as to whether the H^- is bound terminally to the Fe_d atom or forms a bridge between the Fe_p and Fe_d atoms with DFT studies finding that both are feasible.⁷⁷ Figure 8 shows the hydride, H^- , being terminally bound to the Fe_d atom and the [4Fe–4S] cluster in the +1 state; the loss of the hydride as a proton and the proton held by the bridgehead nitrogen to a nearby residue results in a super-reduced, short lived state, H_{sred} which is reported at potentials below –487 mV vs SHE by infrared (IR).⁷⁸ Subsequent oxidation of the [4Fe–4S] cluster and binding of the second

proton to the bridgehead N atom results in the H_{red} state which is the state of the enzyme that reduces H^+ to H_2 . Loss of a proton *via* a proton transfer pathway and oxidation of Fe_d from +1 to +2 state subsequently regenerates the H_{ox} state.⁷⁵

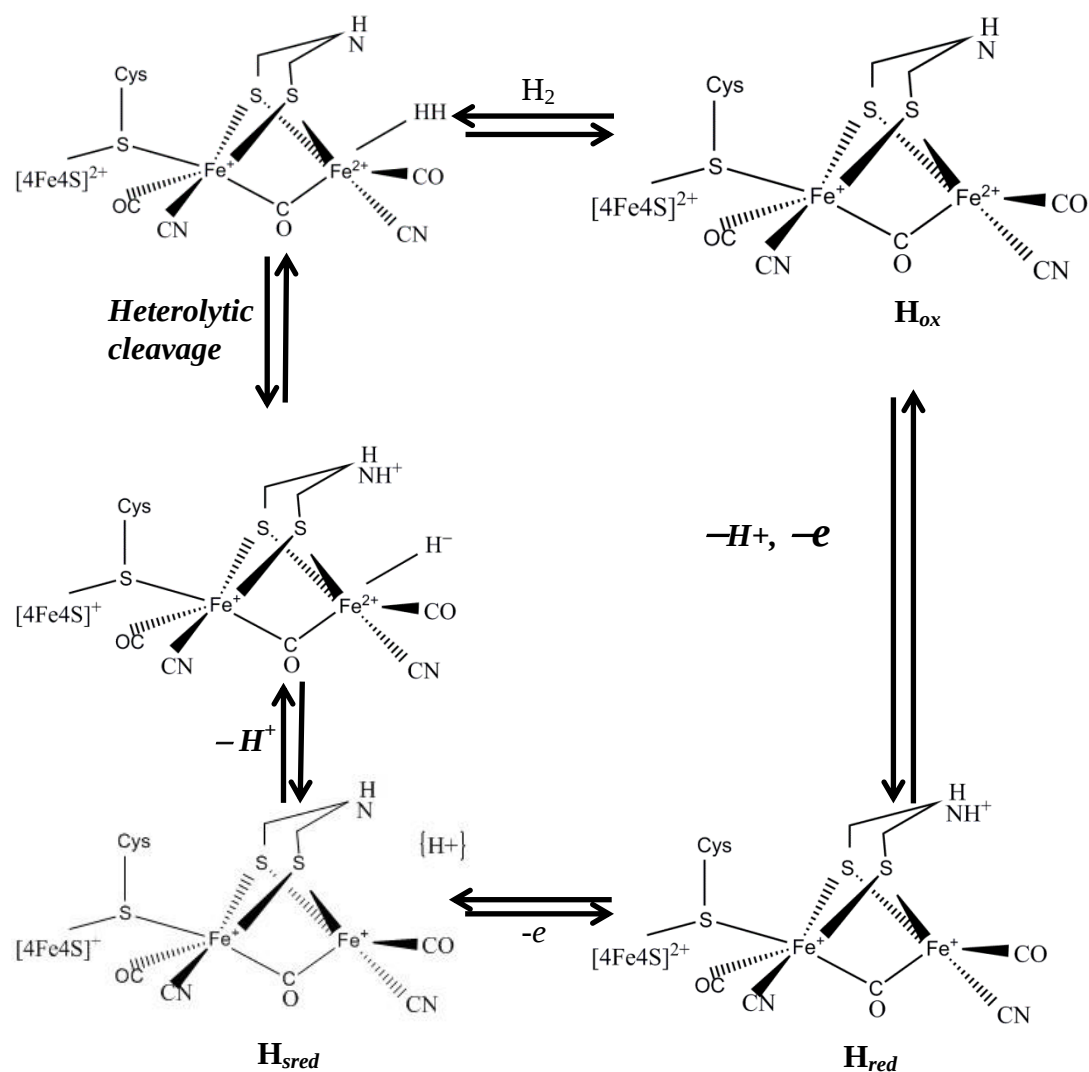


Figure 8: Catalytic cycle of [Fe-Fe] hydrogenase. The mechanism for the catalytic cycle of [Fe-Fe] hydrogenase as proposed by Adamska *et al*⁷⁵ is reproduced here. The substrate (H_2) binds to the H_{ox} state and undergoes heterolytic cleavage with the pendant N and Fe (of the diiron moiety furthest from the [4Fe-4S] cluster) constituting a Frustrated Lewis Pair (FLP) system.⁶⁸ The catalytic cycle involves H_{ox} , H_{red} and H_{sred} states of the enzyme, of which H_{ox} and H_{sred} are EPR-active states.

1.5 Reductive Dehalogenase

1.5.1 Bioremediation of chlorinated substances

Chlorinated solvents observed as contaminants in groundwater include tetrachloroethylene (PCE) and trichloroethylene (TCE) in significant quantities.⁷⁹ Due to their toxicities, intensive research into their removal has been carried out but the methods available, so far, are costly. Innovative treatment methods are continually being explored. Aerobic metabolism (which yields carbon dioxide, water, and chloride) works better with lower degree of chloride substitution while anaerobic metabolism works better with a higher degree of substitution. For highly chlorinated tetrachloroethylene, anaerobic reductive dehalogenation processes brought about by reductive dehalogenases are the only known means of biodegradation.⁸⁰ The terminal reductase in all organohalide respiratory chains is a reductive dehalogenase and understanding the principles in design of them is considered an important goal in achieving bioremediation of PCE and TCE-contaminated water bodies.

Reductive dehalogenation is a two-electron transfer reaction which involves the release of the halogen as an ion and replacement by hydrogen. It is one of the seven (07) ways that dehalogenation is carried out in nature as shown in Table 1. Reductive dehalogenation is thought to either use the organic substrate as the source of both the reducing power and the protons (one-step transfer of two electrons and one proton) or as a source of reducing power only with the proton coming from abstraction from the solvent (two-step reduction process).⁸⁰

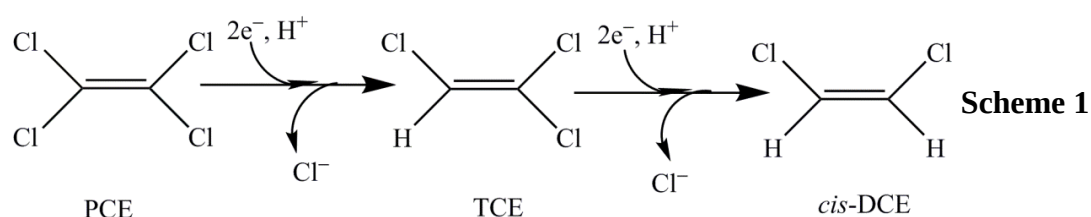
Reductive dehalogenases are found in organohalide respiring bacteria⁸¹ and are members of corrinoids which are vitamin B12-dependent enzymes that are usually

membrane associated and oxygen sensitive.⁸² This makes its study and direct use improbable. They could, however, serve as inspiration for biomimetics for bioremediation purposes.

Table 1: Classification of dehalogenation processes occurring in nature. There are 7 types of dehalogenation known to be carried out by microbes based on the types of bond alteration involved. The table has been adapted from Fetzner *et al.*⁸⁰

Type of dehalogenation	Description
Reductive dehalogenation	halogen substituent is replaced by hydrogen
Oxygenolytic dehalogenation	one (or two) atoms of molecular oxygen are incorporated into the substrate
Hydrolytic dehalogenation	halogen substituent is replaced by a hydroxyl group
Thiolytic dehalogenation	formation of a S–chloromethyl glutathione conjugate
Intramolecular substitution	dehalogenation of vicinal haloalcohols
Dehydrohalogenation	a double bond is formed
Hydration	addition of a water molecule to an unsaturated bond

Scheme 1 is a typical example of a sequence of reactions catalysed by a reductive dehalogenase (PceA dehalogenase which is discussed further in Sections 1.6.2–1.6.4):



1.5.2 PceA dehalogenase from *Sulfurospirillum multivorans*

Sulfurospirillum multivorans (previously known as *Dehalospirillum multivorans*) is an anaerobic organohalide respiring bacterium (an epsilonproteobacterium) which encode for PceA, a non-pseudo vitamin B12-containing reductive dehalogenase.⁸³ PceA

dehalogenase in *S. multivorans* was discovered in cell extracts in 1994 by Neumann *et al.*⁸⁴ This PceA dehalogenase is known to be versatile in the use of substrates but uses mainly tetrachloroethene (commonly known as perchloroethylene, or PCE) or trichloroethene (TCE) as electron acceptors in anaerobic respiration⁸⁴⁻⁸⁵ while hydrogen, formate, pyruvate and lactate can be used as electron donors. Reduced methyl viologen can serve as electron donor for reductive dehalogenation of tetrachloroethene (PCE) and trichloroethene (TCE) and the product of the reductive reaction with either substrates is *cis*-dichloroethene (*cis*-DCE).^{82d}

1.5.3 Structure of the active site of PceA dehalogenase

The structure of the one from *Sulfurospirillum multivorans* have been solved recently (Figure 9A) in which there is a corrinoid (norpseudo vitamin-B12) as the active site (Figure 9B is a Chem Draw representation). The identities of R1 and Y differentiate between the various corrinoids. When R1 is a methyl group, the pseudovitamin cofactor is formed while R1 being a hydrogen makes the norpseudovitamin cofactor. Y can be either CN⁻ or a 5'-deoxy-5'-adenosyl group, thus differentiating between vitamin B12 and coenzyme B12 groups. The corrinoid within the enzyme is in the base-off confirmation⁴⁶ as shown in Figure 9C though that of isolated norpseudo-vitamin B12 is base-on. In base-off configuration, the cobalt is not coordinated to the lower ligand.

There are aromatic residues in the active site which are partially conserved between different reductive dehalogenases but are dispersed in sequence which are thought to play a role in the selectivity shown by the enzymes.⁴⁶ Tyrosine in the 246 position (PceA dehalogenase numbering) is invariant among the different reductive dehalogenation and projects its side chain into the active site; it was found in the crystal

structure of PceA dehalogenase with incorporated TCE that the tyrosine is within hydrogen bonding distance of chlorine when TCE is bound.

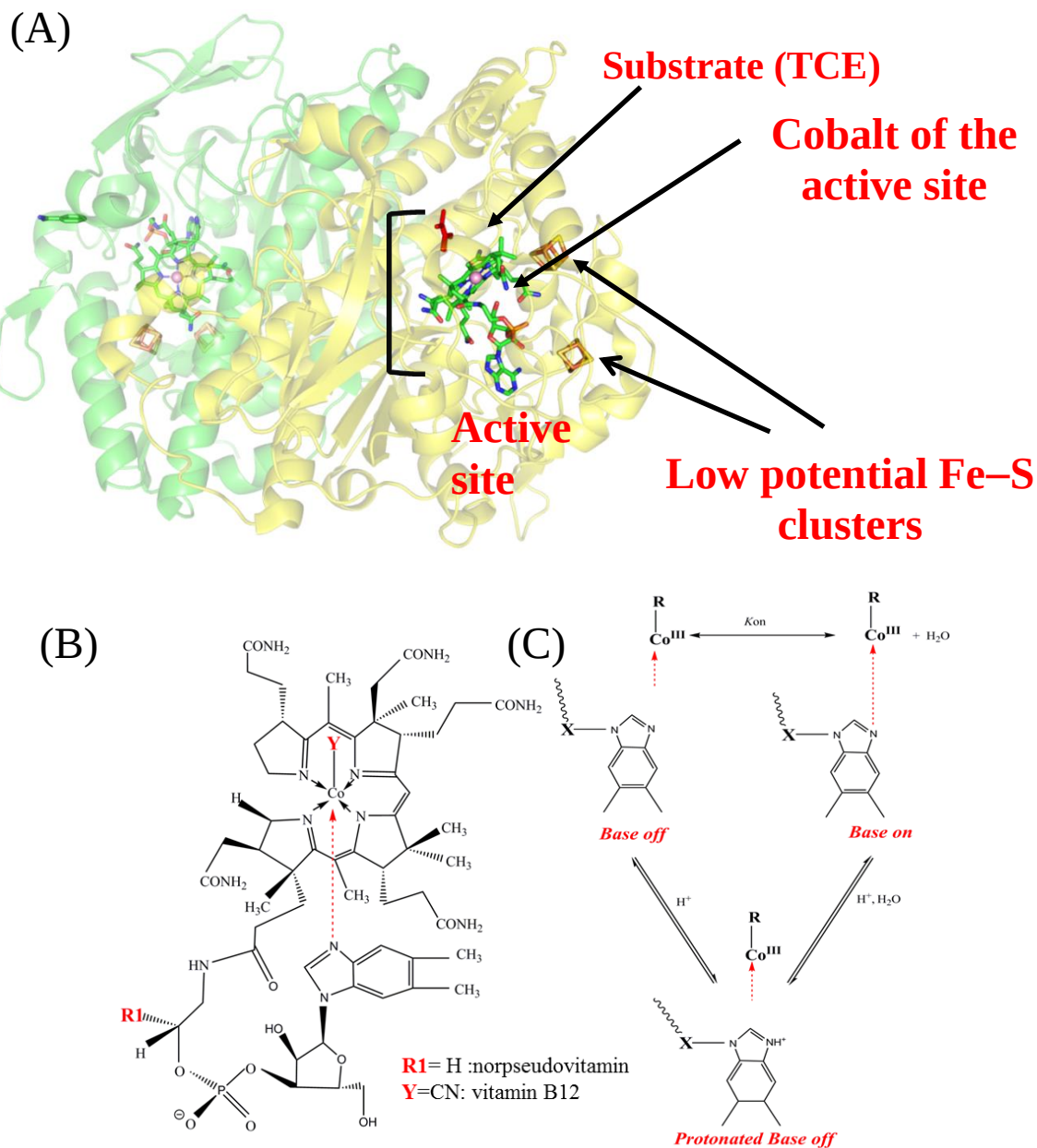


Figure 9: The structure of PceA dehalogenase from *Sulfurospirillum multivorans*. (A) The structure (PDB code: 4UQU) ⁴⁶ is a dimer with one of the sides labelled to identify the active site and the two [4Fe-4S] clusters. The substrate (TCE) is attached at the active site by van der Waals forces and the active site is a norpseudovitamin B12 is a corrinoid. (B) The Chem Draw representation of the norpseudocobalamin is shown. The R1 and Y groups (in red) differentiate between corrinoids. In norpseudovitamin B12, R1 is a hydrogen and Y is a cyanide group. (C) The Chem Draw representations of cobalamin in the base-off and base-on positions are shown. The base-off position can also be obtained when the N in the lower ligand is protonated.

A 12 Å-long channel was identified from the protein surface to the active site and the substrate, either PCE or TCE, has to pass through this channel to get to the active site. Substrate access to the active site is restricted by a gap made up of side chains from the N-terminal and insertion units.⁴⁶ The active site is amphiphilic at the β face of the corrin ring to which access is restricted by a ring of residues which are mainly tryptophan and tyrosine residues.

In the reductive dehalogenase from *Sulfurospirillum multivorans*, two Fe-S clusters lead away from the active site to provide the plumbing/ 'electric wiring' to the surface.⁴⁶ In general, dehalogenases have either two [4Fe-4S] clusters or a combination of one [4Fe-4S] and one [3Fe-4S] clusters leading from the active site to the protein surface. Two [4Fe-4S] clusters exist within *S. multivorans*⁸⁶ with the proximal and distal being positioned at 8.4Å and 9.5Å from the cobalt, respectively. Both the Fe-S clusters are located 6 Å from the surface of the enzyme surface.⁴⁶ The overall structure of PceA dehalogenase is similar to all other reductive dehalogenases except the 3-chlorobenzoate reductive dehalogenases of *Desulfomonile tiedjei* described by Ni *et al* in 1995 which contain a [4Fe-4S] cluster and a [3Fe-4S] clusters.⁸⁷

The native enzyme is encoded with a B protein (with three transmembrane helices) which serves to anchor the enzyme to the exoplasm side of the membrane (Figure 10)–the fact that the enzyme is membrane bound was verified by freeze fracture replica immunogold labelling⁸⁸ although the attachment is probably loose as shearing from the membrane occurs easily during the isolation process of the PceA dehalogenase from the cell extracts.⁸⁹

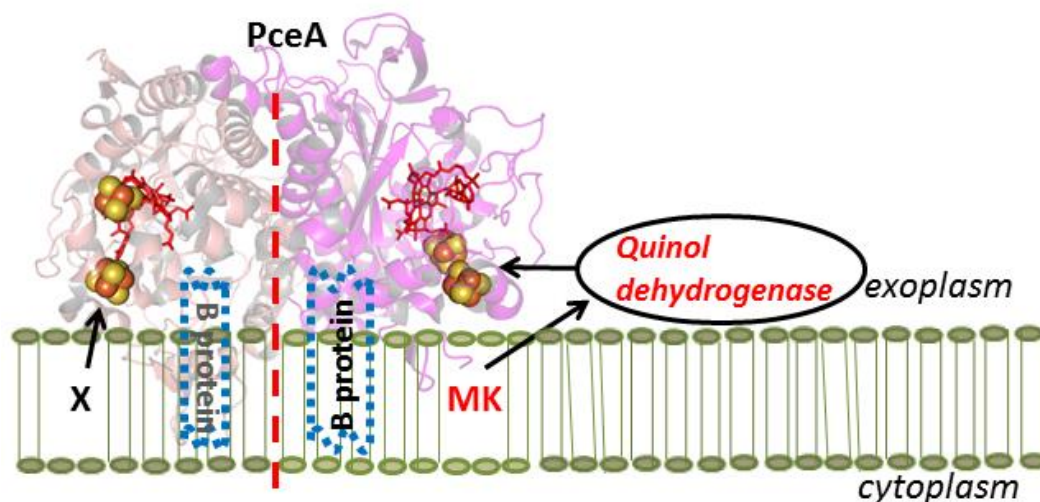


Figure 10: Reductive dehalogenase PceA from *Sulfurospirillum multivorans* (PDB code: 4UQU)⁴⁶ anchored to the cell membrane. Two alternate possibilities are available for the electron shuttling system and are shown on either side of the vertical red dashes. The PceA is anchored to the membrane by a B protein and receives electrons from an unknown component (X) in the membrane. The alternate route of electron transfer to PceA is from a metaquinone system in the membrane (MK) *via* a dehydrogenase.

1.5.4 Catalytic cycle of PceA dehalogenase

The corrinoid active site contains a cobalt (Co) in the catalytic centre, the oxidation states of which maybe +1, +2 or +3.⁹⁰ In the as-isolated form, the active site of the reductive dehalogenase from *S. multivorans* contains cobalt in the Co^{II} state (confirmed by EPR) which is attached non-covalently to the protein scaffold. The redox potential of the cobalt in the the active site was reported as -0.38V vs SHE at pH 7.5⁸⁶ – at least 100mV more positive than the isolated corrinoid,⁸⁶ an influence, in all probability, of the protein scaffold. Since, methyl viologen has been required as mediators in electrochemical experiments, it has long been assumed that Co^{I} is the oxidation state of the enzyme which is catalytically active.^{82b, 91} The Co^{III} state has not been accessed yet even under high potentials, *e.g.*, potentials greater than 150mV vs SHE were applied to membrane-bound PceA of *D. restrictus*.⁹²

The PceA dehalogenase from *S. multivorans* converts PCE to *cis*-DCE via TCE as shown in Scheme 1. There are evidences in support of two mechanisms for formation of TCE from PCE by PceA dehalogenase as outlined below (visualized in Figure 11):

Path One: The cobalt in the +1 state, *i.e.*, Co^{I} , is able to act as a strong nucleophile and attacks the carbon backbone *via* the formation of a cobalt–tetrachlorovinyl complex as an intermediate. The Co–C bond in the resulting cobalt–trichlorovinyl complex then directly abstracts a proton, an electron and releases TCE. Homolytic cleavage of the Co–C bond in the cobalt-trichlorovinyl complex leading to the formation of a trichlorovinyl radical and a Co^{II} is a possibility though not yet proven.

Path Two: The second mode involves the active site acting as the reductant in an electron transfer mechanism. This leads to the formation of a trichlorovinyl radical *via* an intermediate carbanion radical upon elimination of a chlorine. The formation of an cobalt–trichlorovinyl complex through combination of Co^{II} and the trichlorovinyl radical has not been proven,⁹² but cannot be excluded as a possibility. After reduction and protonation of the trichlorovinyl radical the final product TCE is formed.

The reduction step may be brought about by an electron transfer step *via* the cobalt ion in the centre of the active site or *via* the proximal [4Fe–4S] cluster, which is not only located in close proximity to the cofactor (8.4 Å) but is within tunnelling distance of the substrate binding site of the reductive dehalogenase (10.8 Å).⁴⁶ The formation of the *cis*-isomer as opposed to the *trans* form of DCE may be due to the comparably high stability of the *cis*-1,2-dichloroethen-1-yl radical upon TCE dechlorination.⁹³ Whether *via* **Pathway One** or **Pathway Two**, the formation of substrate radical in the catalytic

mechanism was inferred from the detection of adducts with methyl viologen radicals ($MV^{\bullet+}$) and chloropropenyl radicals when dechlorination of chloropropenes was carried out by PceA dehalogenase of *S. multivorans*.⁹⁴ Additionally, the involvement of an external electron transfer as shown in **Pathway Two** is thought to be more favourable than the nucleophilic attack by Co^I based on the steric constraints imposed by the active site as observed in recently obtained structural data.^{46, 95} Another pathway involving the direct interaction of the cobalt ion with the halogen substituent in the substrate has been proposed using evidence from EPR spectroscopy⁹⁵ but further experiments need to be conducted on different reductive dehalogenases with variances in substrate preferences to verify the generality.

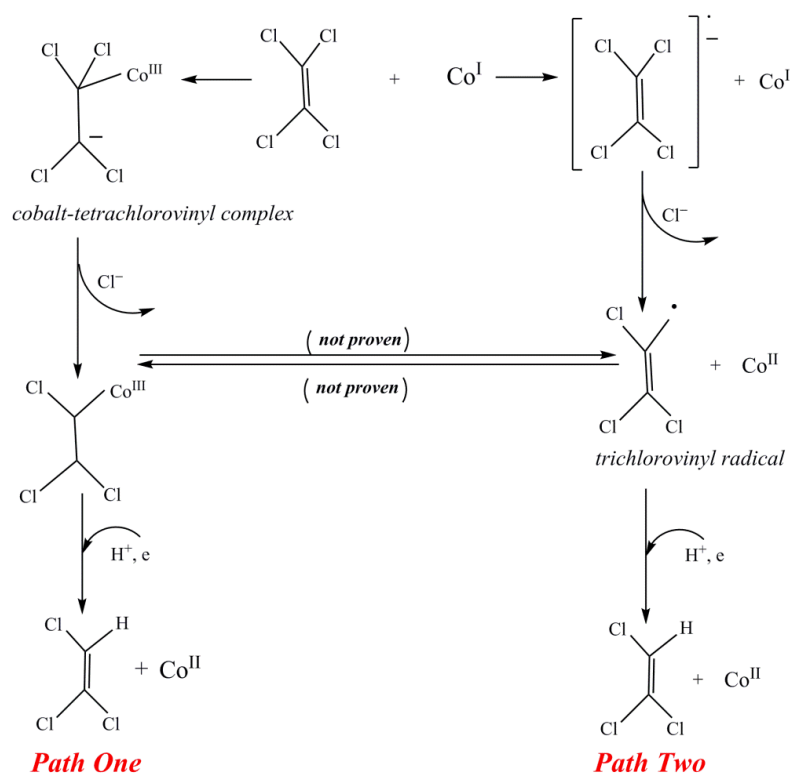


Figure 12: Proposed mechanisms for catalysis by reductive dehalogenase. Path One involves the nucleophilic attack by the Co^I upon the substrate (here, PCE) and formation of a Co^{III} intermediate which subsequently abstracts a proton and an electron to release TCE. Path Two involves the cobalamin acting as the reductant in an electron transfer mechanism and leading to the formation of a trichlorovinyl radical *via* an intermediate carbanion radical. The radical then receives a second electron and proton to release TCE.

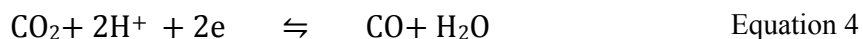
1.6 Carbon Monoxide Dehydrogenases

1.6.1 CO/CO₂ interconversion by CODH

Carbon dioxide (CO₂) is a known air pollutant⁹⁶ and thus a clean, efficient conversion of CO₂ to a less harmful form, particularly into carbon-based fuels is an important scientific quest. However, carbon dioxide is a difficult molecule to activate and intensive research is ongoing to understand CO₂ activation in order to understand the principles involved.⁹⁷

Carbon monoxide (CO) participates in a broad range of processes⁹⁸ and, for some microbes, CO is a source of carbon and energy.⁹⁹ Fisher –Tropsch processes are based on CO with CO and H₂ forming a feedstock mixture known as syngas for the formation of liquid fuels. Thus an alternative to conversion of CO₂ to fuels such as methanol can be conversion to CO, an industrially relevant molecule.

It has been inferred in various studies that CO levels in the early atmosphere were probably near 100 ppm, though prevailing readings of atmospheric concentrations are between 0.05–0.35 ppm.^{98, 100} It is probable that nature has found a way to utilize CO in the atmosphere and an investigation of the microbes has revealed carbon monoxide dehydrogenases (CODHs) with high affinity for CO and which are able to catalyse CO₂/CO interconversion as shown in the following equation:¹⁰¹



The interconversion brought about by CODH occurs quite efficiently in nature and understanding how it achieves such is important for designing the best catalysts for CO₂ reduction.¹⁰² Protein film electrochemistry (PFE) has recently been used extensively to

study carbon monoxide dehydrogenases-I and II from *Carboxydotherrmus hydrogenoformans* (CODH I_{Ch} and CODH II_{Ch}) especially their interactions with substrate analogues which often are also its inhibitors.¹⁰³ The inhibition brought about cyanide (HCN/CN⁻) and cyanate (NCO⁻) has been shown to be potential dependent as they selectively target the different redox levels of CODH (Section 1.7.4).^{103a, 103c} Interestingly, the active site of Ni-CODH is also capable of processing nitrogen based molecules as exemplified by PFE studies of nitrous oxide, N₂O,^{103b} and spectroscopic studies of hydroxylamine, NH₂OH,¹⁰⁴ as substrates.

1.6.2 Classification of CODH

There are two types of CODHs: aerobic and anaerobic CODH.^{103c} Aerobic CODH contains a Mo-Cu active site¹⁰⁵ while the anaerobic contains a unique Ni containing active site as shown in Figure 13. The K_M^{CO} of aerobic CODH is $\sim 10 \mu\text{M}$ ¹⁰⁶ while for anaerobic CODH, it is higher (30 μM for CODH I_{Ch} and 18 μM for CODH II_{Ch}).^{99, 107} The k_{cat} is also higher for the anaerobic CODH: a value as high as 40000 s⁻¹ at pH 8, 70°C has been reported for the Ni-containing CODH^{99, 108} while the highest for Mo-Cu containing CODH is approximately 100 s⁻¹.^{106, 109} The high activities of Ni-CODH are the reason for invested research in them despite their O₂-sensitivity. The O₂-sensitivity of these enzymes mean that they themselves may not be used in technological applications, but they do provide excellent basis for understanding the ‘*design principles*’ involved in achieving such high turnover rates. The research into Ni-CODH is thus usually two-pronged: 1. elucidation of its high activity and 2. understanding of its O₂-sensitivity to avoid them in designing biomimetics based on CODHs.

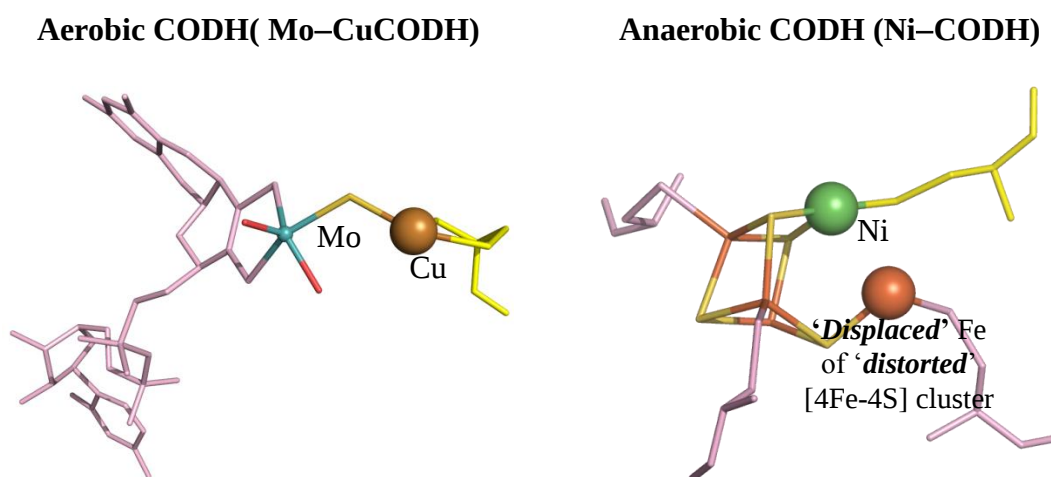


Figure 13: Pymol representations of active sites of Mo– Cu containing and Ni– containing CODH. The Mo–Cu active site is present in CODH from aerobic organism such as *Oligotropha carboxidovorans* (PDB code: 1N63)¹¹⁰ while the Ni– containing CODH is present in anaerobic organisms such as *Carboxydothemus hydrogenoformans* (PDB code:1SU7)¹².

1.6.3 Structure of the active site of Ni–CODH

The X-ray crystal structures of carbon monoxide dehydrogenase II from *Carboxydothemus hydrogenoformans* (CODH II_{Ch})^{12, 111} advocate that enzymes of the anaerobic Ni–containing CODH class (Ni– CODH) are homodimeric with each subunit containing a unique [Ni–4Fe–4S] cluster known as the C cluster, a [4Fe–4S] cluster known as the B–cluster and another [4Fe–4S] cluster known as the D–cluster which it shares with the alternate subunit. It is the D–cluster that communicates with the external ferredoxin partner.¹⁰² In the C–cluster, the Ni–atom is coordinated to two S atoms of a cubane-like [3Fe–4S] structure in which there is a ‘displaced’ 4th Fe atom as highlighted in Figure 13. A fifth sulfido ligand bridging the dangling Fe and Ni atom has been reported though not conclusively proved in active enzymes¹¹² and hence is not shown in Figure 13.

1.6.4 Catalytic cycle of Ni–CODH

During catalysis, Ni– CODH is known to cycle through three redox states, namely, C_{ox} , C_{red1} and C_{red2} , of which C_{red1} and C_{red2} are catalytically active. The redox transformations have been studied by EPR titrations. The inactive oxidized state C_{ox} is EPR silent as studied with a CODH complex from the anaerobic bacterium *Moorella thermoacetica* (CODH_{Mt}). Feng and Lindahl characterized the oxidized C_{ox} state as $[Ni^{2+}Fe^{3+}]:[Fe_3S_4]^{-1}$ and reported it as being formed at -150 ± 50 mV vs SHE when studying CODH from microbe *Rhodospirillum rubrum* (CODH_{Rr}).^{113 114} This value, however, contrasts study of the same enzyme by Heo, Halbeib and Ludden who calculated -316 mV as the value.¹¹⁵

The C_{ox} state is reduced by one electron to yield the oxidized active state known as C_{red1} which exhibits a characteristic EPR signal with $g_{av} \sim 1.82$ and which reacts rapidly with CO to be converted to a 2-electron reduced state known as C_{red2} ($g_{av} \sim 1.86$). The reduction potential for the two-electron interconversion between C_{red1} and C_{red2} as reported by studying CODH_{Mt} is approximately -520 mV at pH 7 and is very close to the formal reduction potential for the CO_2/CO couple.³³ The electron distribution in the C–cluster in these different oxidation levels is still unresolved though it may be inferred, based on the large ^{57}Fe – and minimal ^{61}Ni –hyperfine values, that most of the unpaired spin density is localized on the Fe atom in both the C_{red1} and C_{red2} states.¹¹⁶

The basic mechanistic model for CO_2/CO interconversion is given in Figure 14. Jeoung and Dobbek formed crystals of CODH II_{Ch} with $NaHCO_3$ (to serve as source of CO_2) under reducing conditions (-0.6 V) and revealed an ‘intermediate’ state in which CO_2 is bound through the C–atom to the Ni atom to complete a square-planar coordination

geometry typical of Ni^{2+} while the O-atom is bound to the displaced Fe.^{111c} Thus, the role of the displaced or dangling Fe atom during CO_2 reduction is assumed to be the abstraction (as hydroxide) of the O-atom of CO_2 . A proton-coupled two-electron reaction is then thought to occur resulting in CO bound to the Ni atom.

During the reverse reaction of CO oxidation, the Fe atom (attached to a hydroxyl group) is assumed to be involved in nucleophilic attack on the Ni-bound carbonyl-C atom. The similar EPR spectra for the C-cluster in the two states ($g_{\text{av}} < 2$) suggest that the same $[\text{Fe}_3\text{S}_4]^{1-}$ fragment is present in both C_{red1} and C_{red2} states¹¹⁶; therefore, the two-electron transition might occur *via* $\text{Ni}(0)$ or a Ni^{2+} -hydride intermediate state.¹¹⁷

In PFE experiments, the interconversion between the various redox states is achieved by changing the applied potential to the system.¹⁰³ For example, C_{ox} -dominated CODH I_{Ch} and II may be formed by maintained a high potential (> -50 mV vs SHE) while C_{red1} -dominated CODH I_{Ch} and II forms by maintaining applied potentials between -0.52 V to < -0.05 V vs SHE. The catalytically active state for CO_2 reduction, C_{red2} , is achieved by application of potentials below -0.52 V vs SHE, with -0.76 V vs SHE being the default applied value in chronoamperometry experiments which require the maximum driving force when using PGE electrodes in PFE experiments.

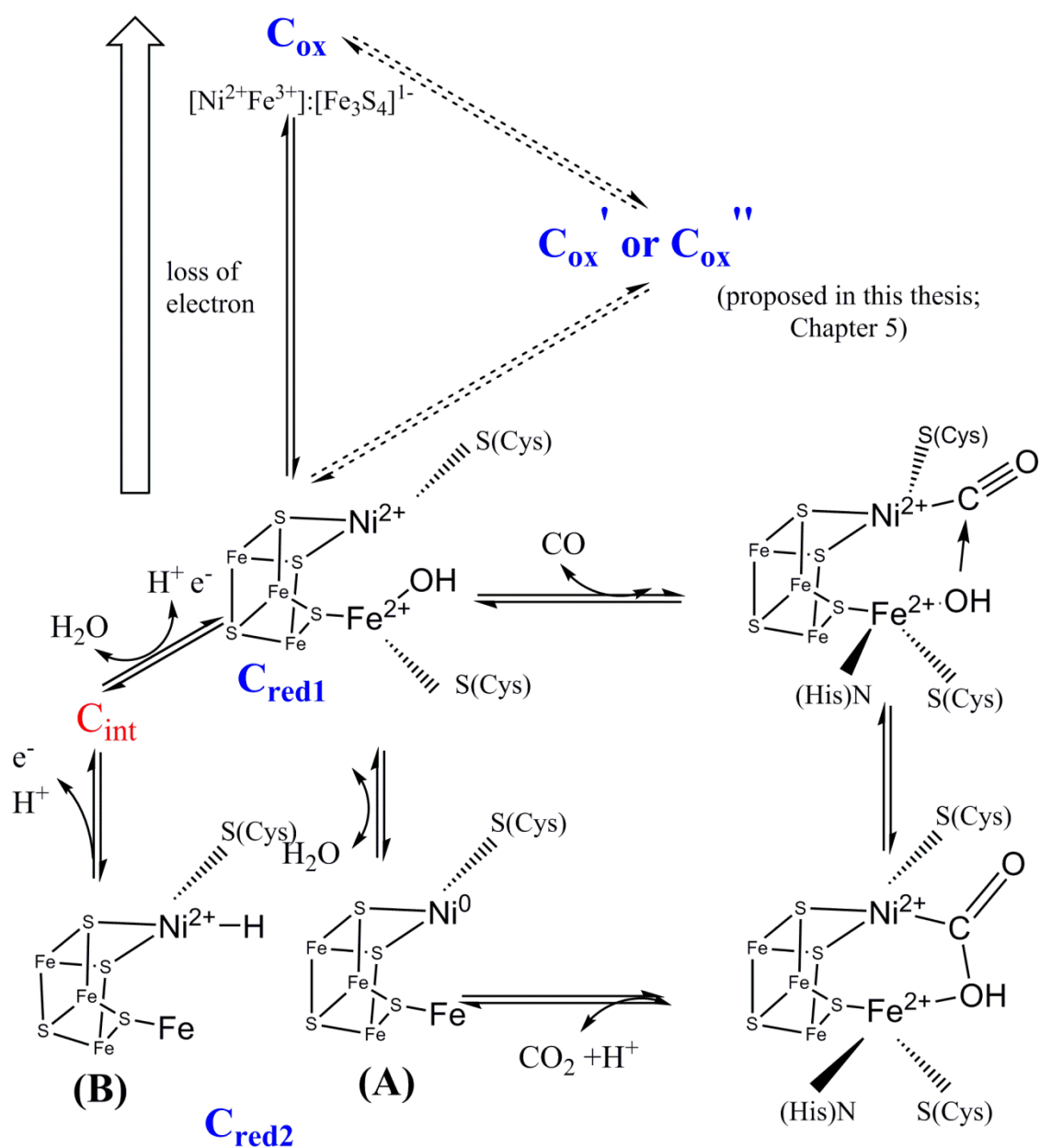


Figure 14: Proposed catalytic cycles of Ni-CODH. The figure shows two probable pathways for the formation of C_{red2} . (A) The Dobbek and Lindahl pathway envisions the '0' state for the Ni after receipt of two electrons as CO binds and is released as CO_2 ^{111c 118} while (B) Fontecilli-Camps¹¹⁷ pathway involves the formation of a hydride at the Ni atom. The C_{int} state has been proposed as requirement for long-range electron transfer. This figure is modified (with permission) from a study on CODH by PFE as published by Wang *et al.*^{103b} Hypothetical states (C'_{ox} and C''_{ox}) which are discussed in Chapter 5 are shown here.

1.7 Aims and Structure of this thesis

Several Fe–S containing enzymes were investigated in this thesis to elucidate their catalytic properties. The PFE technique was utilized primarily though other techniques such as site-directed mutagenesis (SDM) and electrochemical impedance spectrometry (EIS) were exploited to enable further probing of these enzymes. While Chapter 1 served to introduce the enzymes and provide the background for the studies undertaken during the DPhil programme, Chapter 2 shall highlight the theories that underline the various techniques that were utilized. The remainder of the thesis is arranged in terms of the investigations carried out to realize the aims envisioned for the various projects:

- The first aim of this thesis was set to be the investigation of the role of proline residue at position 508 of *Escherichia coli* Hyd–1 to an alanine residue by using site-directed mutagenesis (SDM). Given the role arginine at the 509 position is thought to play in H₂ activation at the Ni–Fe bimetallic active site of Hyd-1,⁴⁴ the study of the role of the proline residue in stabilization of the arginine is significant to enable proper design of biomimetics based on the Ni–Fe bimetallic active site. These biomimetics are indispensable in enabling H₂ economy. The results of the SDM process and subsequent characterization by PFE to evaluate the impact of the mutation on the functioning of the enzyme are given in Chapter 3.
- Reductive dehalogenase PceA from *Sulfurospirillum multivorans* has not yet been characterized by direct electron transfer with an electrode. Based on the success of PFE in studying other membrane-bound enzymes on PGE electrode, the second aim of this study was set to characterize the PceA enzyme on PGE electrode by PFE. Since the substrates of PceA are known water pollutants and the enzyme is considered to have potential in bioremediation applications, it was determined that studies shall be conducted into action of some inhibitors and their operational

potential window. The results of the investigations of PceA by PFE are given in Chapter 4.

- CODH is an important and highly efficient enzyme involved in CO_2/CO conversion and is an excellent basis for inspiration of industrially important biomimetic catalysts for CO_2 reduction. Since PFE has proven to be an apt technique for investigating inhibitor interactions with CODH, it was decided to utilize PFE for studying the enzyme's interaction with potent oxidants, O_2 and H_2O_2 , as inhibitors of the enzyme which prevent their direct use in technology. Furthermore, it was decided to utilize PFE to study the enzyme's interaction with another reported substrate, hydroxylamine (NH_2OH), which is nitrogen-based. Interestingly, NH_2OH is analogous in structure to H_2O_2 , an inhibitor. The results of the various experiments are expounded in Chapter 5.
- Electrochemical Impedance Spectrometry (EIS) can be utilized as a probing technique for gaining insight into time-dependent and independent components of enzymes. Two $[\text{Fe}-\text{Fe}]$ hydrogenases, *CpI* and *CrHydA1*, with identical active sites but with and without Fe-S cluster respectively could be studied with EIS to differentiate signals from an electron relay (which may be proposed to be resistor-like) from those arising from the active site (which may be proposed to be capacitive in nature). The results of the use of EIS on *CpI* and *CrHydA1* are given in Chapter 6.

Chapter 2: Electrochemical and Molecular Biology Theories

2.1 Introduction to Utilized Electrochemical Techniques

Protein film electrochemistry (PFE) is a suite of dynamic electrochemical techniques which can be applied to the study of enzymes with redox-active centres.³¹ The PFE procedures generally involve loading an aliquot of the enzyme (in picomole range) onto Pyrolytic Graphite Edge (PGE) electrodes before carrying out cyclic voltammetry (CV) or chronoamperometry (CA) experiments.^{28, 30} The attachment of the enzymes to the electrodes is mainly *via* van der Waals forces though the attachment may be reinforced with the use of polymixin β –sulfate, neomycin and tobramycin as co-adsorbates.¹¹⁹ The use of these co-adsorbates is recommended for use with enzymes with a net negative surface charge (as in the case of CODH); their positively charged amino groups form ternary salt bridges between the protein and the negatively charged electrode surface. Enzymes not requiring the presence of co-adsorbates generally have net positive surface charge and/or are at least partially hydrophobic. As a general rule of thumb, enzymes which are membrane-bound in their physiological conditions respond well to application onto PGE electrodes. The covalent attachment of enzymes to PGE electrodes improves the signal-to-noise ratio; this attachment could be brought about by addition of carbon nanotube layers to the electrode to which the enzymes may be covalently linked.¹²⁰

Since electrons need to be transferred to within the enzyme from the electrode or from the enzyme to the electrode to generate signals in the form of currents (Section 1.2), it is imperative that electron-transferring constituents within the enzyme, *e.g.*, the Fe–S electron relay, are located within electron tunnelling distance from the surface. If the active site itself is within tunnelling distance, then the presence of the electron relay is not required.

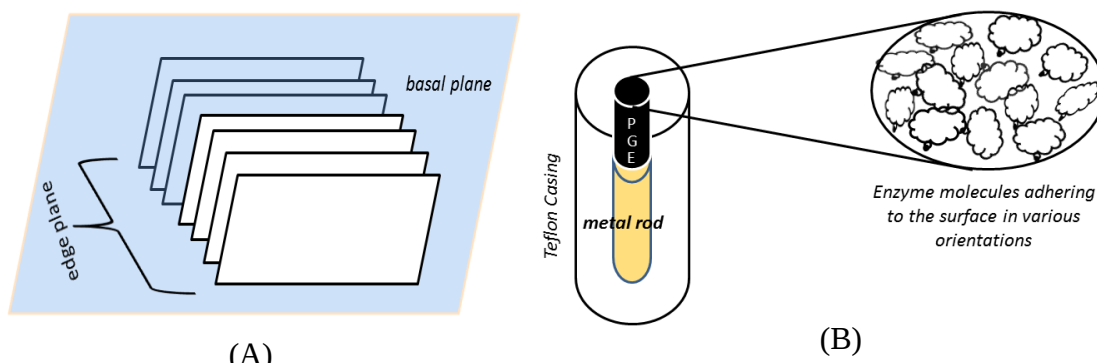


Figure 15: Structure of highly ordered pyrolytic graphite and PGE electrode. (A) This panel shows the two types of graphite surfaces: basal and edge. It has been found that the edge is more conductive than the basal and is thus used in PFE experiments. (B) Before each experiment, the surface is roughened and the enzyme solution spotted onto the electrode surface by pipetting up and down several times. The enzyme molecules do not orient themselves similarly on the surface and those with electron accepting/donating groups within electron tunnelling distance of the PGE surface are able to generate signals as current.

It has been shown that adhesion of proteins occur more strongly to the edge plane of pyrolytic graphite as opposed to the basal plane (Figure 15A).⁵⁰ Before each experiment, the surface of the PGE is abraded with sandpaper or other coarse surfaces *e.g.*, alumina to expose fresh surfaces.¹²⁰⁻¹²¹ The surfaces of the PGE tip provides a rough surface with unsatisfied carbon atoms which react with water /O₂ to give polar functional groups such as C = O, C–OH and COOH which aid in the subsequent binding of enzyme molecules. Additionally, the formed grooves increase the probability that enzyme molecules are in various orientations which, in turn, enhance the probability of better electrical contacts between the enzyme and the electrode surface (Figure 15B). Throughout the work presented in this thesis, polishing was undertaken with P400 Tufbak Durite sandpaper.

The ability of PGE electrodes to maintain electrical contact as occurs *in vivo* forms the basis of success of PFE: recalling hydrogenases-1 from *E. coli* are attached to membrane *via* b-cytochrome (HybC) and HyaA contains an α -helix attachment to the

membrane (Section 1.5.3), it may be noted that COO^- species on the PGE surface may mimic the charged surface of the b-cytochrome, and the hydrophobic basal plane cavities may help to anchor the enzyme molecules as the α -helix does to the lipid core.³⁰

When the enzyme is adsorbed onto the PGE surface and substrate molecules are present, electrons transfer to and from the electrode due to catalysis by the enzyme. Protein film electrochemistry focuses entirely on net catalytic electron flow that is observed and recorded as direct current (DC) in voltammograms.^{28-29, 32} This current-voltage relationship may then be analysed to learn about the workings of the enzyme under study. The magnitude of the current is proportional to the electron flow which corresponds directly to the number of substrate molecules being processed by the enzyme; the exact value of the turnover frequency is knowable when the number of enzymes molecules on the surface can be calculated.^{28, 30}

For an electrochemical system, application of an alternating voltage generates an electrical response in the form of alternating current (AC) depending on the texture and nature of electrodes, microstructure of the electrolyte and species of charge present.³⁵ Figure 16 shows the relationship between a sinusoidal AC response to a voltage applied in a sinusoidal manner with a fixed amplitude and a certain frequency. The current lags behind the voltage by a phase shift known as the time lag. Similar to PFE experiments, the analysis of this current-voltage relationship provide information about the system under study.

In PFE experiments, the resistance (R) offered by a circuitry is defined as the ratio between applied voltage (V) and current (I) as per Ohm's law ($R = V/I$). However, the

AC technique of electrochemical impedance spectroscopy (EIS) is based on an analogue of Ohm's law as given below:³⁵

$$\tilde{V} = \tilde{I}Z \quad \text{Equation 5}$$

where Z is the impedance (generalized form of resistance), and the over-bars imply quantities which are time-dependent. The voltage, $\tilde{V}$, is given by

$$\tilde{V}(t) = V_0 \cos(\omega t) = V_0 \exp[j(\omega t)] \quad \text{Equation 6}$$

where, ω is the perturbation frequency, V_0 is the amplitude of the AC signal and $j = \sqrt{-1}$. Generally, a sufficiently small voltage modulation is used to maintain a nonlinear relationship between the applied potential and the observed current. The current, $\tilde{I}$, lags behind the voltage by a phase shift given by ϕ (time lag) and, is given by the following relationship:

$$I(t) = I_0 \cos(\omega t - \phi) = I_0 \exp[j(\omega t - \phi)] \quad \text{Equation 7}$$

where I_0 is the amplitude of the sinusoidal current response (obtained by an analyzer).

Thus, Z , the impedance, is given by

$$Z(t) = \frac{V(t)}{I(t)} = \frac{V_0 \cos(\omega t)}{I_0 \cos(\omega t - \phi)} = \frac{V_0 \exp[j(\omega t)]}{I_0 \exp[j(\omega t - \phi)]} = Z_0 \exp[j\phi] \quad \text{Equation 8}$$

where Z_0 is the amplitude of impedance of the system. This impedance, Z , has time-independent and time-dependent components which vary according to the magnitude of ϕ . The technique where impedance is varied and measured across a range of frequencies in an electrochemical system and utilized to learn more about the system is known as Electrochemical Impedance Spectroscopy (EIS).

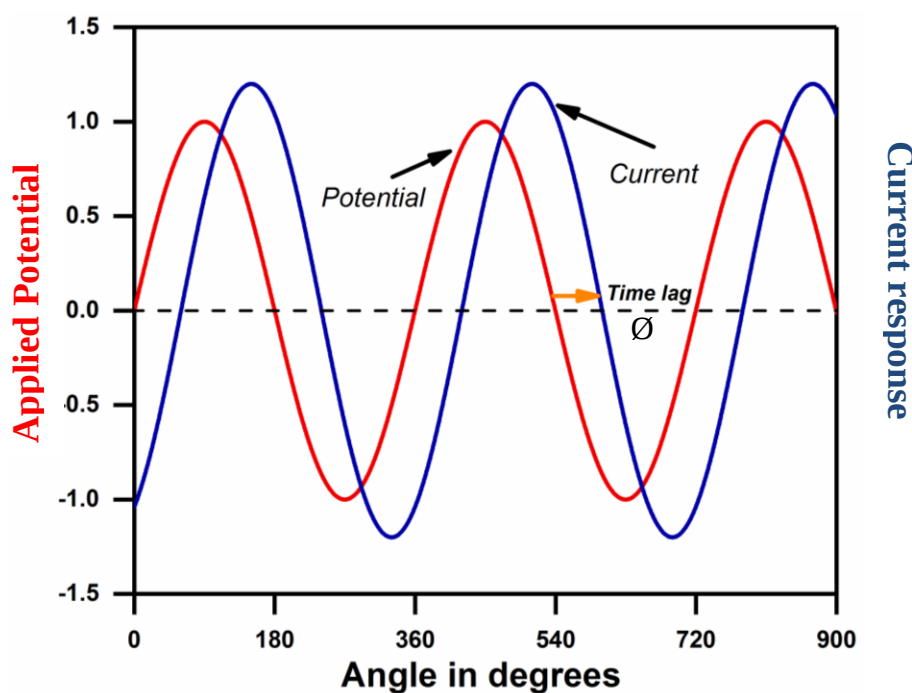


Figure 16: Sinusoidal voltage applied to a sample and current response obtained in impedance measurements. When a voltage of certain amplitude and frequency is applied in a sinusoidal manner, it creates a time-dependent current as a function of the applied frequency. The induced current is out of phase by a time lag, ϕ , and is generally of a different amplitude. Adapted from MacDonald.³⁵

2.1.1 Three-Electrode Electrochemical Set-ups

A three-electrode set-up for PFE and EIS measurements was used in this thesis: the working electrodes were made of PGE constructed in-house.⁵⁰ The quantity of enzyme spotted onto the electrode per run is in the sub-picomolar range making it ideal as a technique to studying enzymes whose harvesting requires considerable time and effort.²² A three-electrode system comprises of the following: a working electrode, a counter electrode and a reference electrode immersed in electrolytic solution as shown in Figure 16. The potential applied is then a sum of the following:

$$Q\Phi_{w/s} + Q\Phi_{s/r} + iR$$

where, $Q\Phi_{w/s}$ represents the potential difference between the working electrode and solution, and $Q\Phi_{s/r}$ represents the potential difference between the solution interface and the reference electrode.

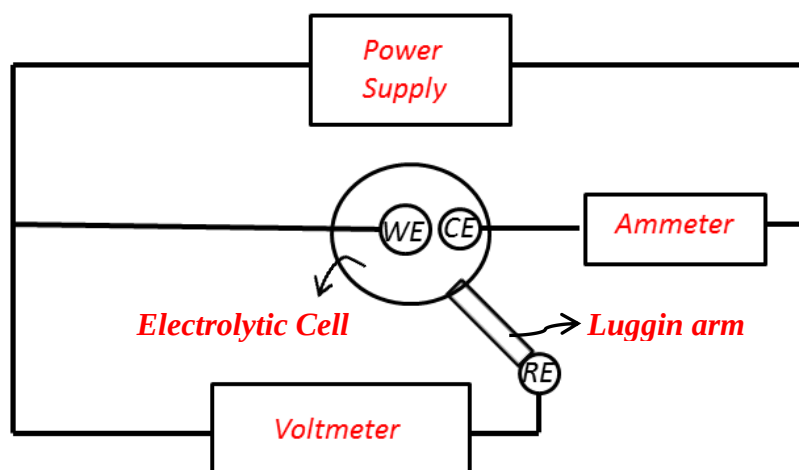


Figure 17: The three electrode set-up used in PFE experiments. WE stands for the working electrode and CE stands for the counter electrode. They are placed within the same electrolytic cell which is connected *via* a Luggin arm to the reference electrode, RE. The luggin arm prevents mixing of the electrolytic solution with that of the 0.1 M NaCl solution in which RE is immersed to provide non-isothermal reference. Generally, WE are PGE electrodes constructed in-house and CE is a Pt wire.⁵⁰ The RE is generally a standard calomel electrode (SCE). The voltmeter, ammeter and power supply are all provided by a potentiostat when carrying out PFE experiments- it provides the desired voltage or a range of voltages for the experiments.

The term iR arises from the resistance experienced by the current passing between the working electrode and the reference electrode. By using a highly conductive Pt wire as a counter electrode, the iR becomes negligible as very little current is passed through the high impedance reference electrode (placed in a Luggin arm) and renders the term $Q\Phi_{s/r}$ constant. The applied potential is then simply equal to the term $Q\Phi_{w/s}$ which is the potential existing at the working electrode.¹²²

2.1.2 Basis for PFE measurements: Cyclic Voltammetry (CV)

This technique is an expanded version of application of linear sweep voltammetry (LSV) in an electrochemical set-up whereby the applied potential is varied linearly between two potentials measured in volts (E1 and E2). The variation in between the potentials is time dependent (measured in seconds) and is known as the scan rate, v , where

$$v = \frac{(E_2 - E_1)}{time} \left(\frac{V}{s} \right) = \frac{\delta E}{\delta t} \quad \text{Equation 9}$$

In cyclic voltammetry (CV) experiments, the applied potential is swept from E1 to E2 and then back to E1 to link electron flow to the reaction conditions. This voltammetry technique makes a very important assumption: the oxidized and reduced species are both strongly adsorbed and have the same enthalpy of adsorption.³⁵ Use of cyclic voltammetry as opposed to single potential sweep alone helps to distinguish rapid, steady-state catalytic activity at different potentials from relatively slow, potential-dependent changes in activity that are revealed as hysteresis (the non-overlapping between the forward and reverse scan).²⁸

2.1.2.1 Non-faradaic signals

The current that arises in PFE measurements in the absence of enzyme catalysis is called **non-Faradaic current**. It arises from the current which serve to compensate for the charge that builds up on the electrode surface from the rearrangement of ions in the solution at the electrode interface under an applied potential (constituting a double layer). The double layer acts as a capacitor (C_d) which charges and discharges, the magnitude of which depends on the surface area of the electrode by which this double layer forms (higher surface area leads to larger signals) and composition of the bulk

solution. This impacts the non-Faradaic current (i_{nf}) as does the scan rate (v) (faster scan rate leads to a larger signal) and thus, i_{nf} is given below as:

$$i_{nf} = C_d \frac{\delta E}{\delta t} = vC_d \quad \text{Equation 10}$$

A blank scan using the bare electrode is recorded before each experiment (a typical one is shown in Figure 18): conditions (such as pH, temperature, composition) between the experimental run and that of the blank scan are kept constant other than the variable under study.

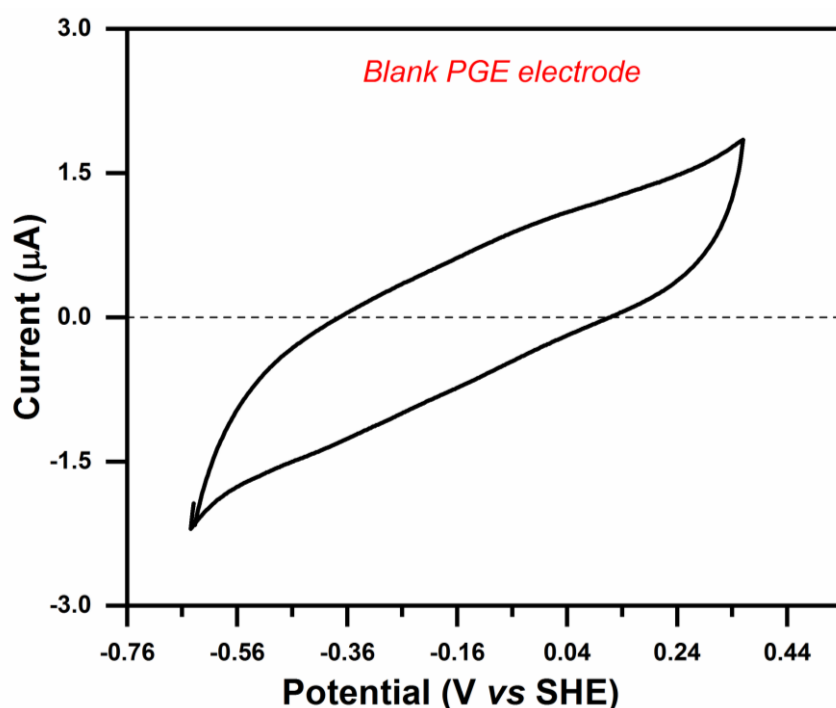


Figure 18: Typical blank scan of a PGE electrode. The shape is obtained by a running a CV with the PGE electrode, with or without enzyme-adsorption, dipped into the electrolytic solution. Ideally, the shape and magnitude of the current profile does not change upon enzyme adsorption onto the electrode. Upon catalysis, a very different current profile is obtained (see Figure 19) and though direct subtraction cannot take place due to changes in the surface between the blank electrode and that with adsorbed enzyme carrying out catalysis, a good indication of the enzyme signal in relation to electrode capacitance is obtained.

2.1.2.2 Faradaic signals (Turnover signals)

The signal obtained upon enzyme catalysis is called a **Faradaic current**. A typical catalytic profile is shown in Figure 18 as a black trace.

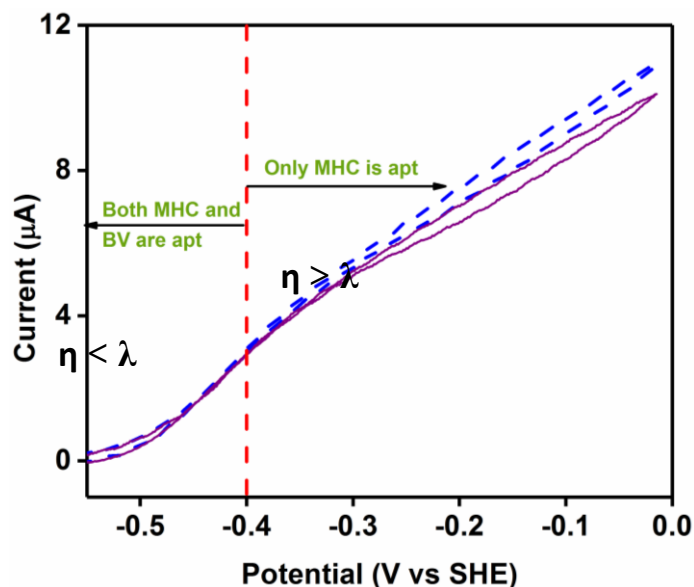


Figure 19: A typical catalytic profile with areas of applicability of the Butler-Volmer (BV) and Marcus-Hush -Chidsey (MHC) theory. The solid black trace is that of a typical catalytic profile obtained when an enzyme-adsorbed PGE electrode is immersed in electrolytic solution with substrates present. Mass transfer is assumed to be non-limiting and inhomogeneity in the orientations of adsorbed molecules negligible (see text). The figure also shows a residual slope (blue dashes) which is obtained in case of considerable inhomogeneity in the orientations of adsorbed enzyme molecules as a black dotted slope.¹²³ Various theories exist to explain the catalytic profile based on interfacial electron transfer of which Butler-Volmer and Marcus-Hush -Chidsey (MHC) theories are relevant to this thesis. When the reorganization energy of the system is high compared to the overpotential being applied ($\lambda > \eta$), Butler-Volmer theory is adequate while at higher values of overpotential ($\lambda < \eta$), the Marcus theory is more applicable.¹²²

Faraday's law of electrolysis gives

$$i = AFj \quad \text{Equation 11}$$

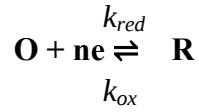
for a redox reaction where a species is either oxidized or reduced at the electrode surface and in which A is the electrode area, F is the Faraday constant and j is the flux of electrons reaching or being withdrawn from the electrode surface $\left[\frac{\text{moles}}{\text{s (unit area)}}\right]$.¹²² If the

species at the electrode surface is being reduced and j_{red} is the flux of electrons bringing about the reduction process, then Equation 11 maybe re-written to give

$$i_{red} = AFj_{red}$$

where j_{red} is a function of the applied potential.

Alternatively, for a reaction



where k_{red} and k_{ox} are the rate constants for oxidation and reduction respectively, j_{red} , is then given by Equation 12: ¹²²

$$j_{red} = -nk_{red}\Gamma_{ox} \quad \text{Equation 12}$$

where Γ_{ox} is the electroactive coverage of the oxidized species, n = number of electrons involved in the chemical reaction and the $-ve$ sign indicates a reductive current. Thus, the reduction current at a particular potential can be obtained by combining Equation 11 and Equation 12 to give

$$i_{red} = -AFnk_{red}\Gamma_{ox} \quad \text{Equation 13}$$

Similarly, an oxidation current at a particular potential is given by

$$i_{ox} = AFnk_{ox}\Gamma_{red}$$

Thus, at a particular potential, the total current (i) is then given by

$$i = i_{ox} + i_{red} = AFnk_{ox}\Gamma_{red} - AFnk_{red}\Gamma_{ox} \quad \text{Equation 14}$$

Equation 14 forms the theoretical basis of deriving the total current (i) at any particular potential.

The applied potential is an important aspect of PFE experiments as it relates to the electron transfer that occurs across electrode-electrolyte interfaces. Two theories, as it relates to this thesis, are the Butler-Volmer (BV) and Marcus theories and are discussed below. Another aspect of the catalytic graph where increased values of applied potential results in a slope in the graph, as modelled by Leger *et al*¹²³, is also summarized.

Butler-Volmer theory:¹²² The rate constants for reduction and oxidation, k_{red} and k_{ox} , are terms that can be related individually to rates of electron transfer at equilibrium potential according to the Butler-Volmer equations given in Equation 15 and Equation 16. The rate constants at equilibrium potential are the standard electron exchange rate constants for reduction and oxidations, k_{red}^0 and k_{ox}^0 , respectively and the applied overpotential, η .¹²² According to BV theory, it is predicted that k_{ox} and k_{red} increases exponentially with $\eta = (E - E^{o'})$ where E is the applied electrode potential and $E^{o'}$, is the equilibrium potential (the potential established under standard conditions by the mixture of oxidized and reduced species).

At zero overpotential, *i.e.*, at equilibrium potential, the standard electron exchange rate constants are equal to one another ($k_{red}^0 = k_{ox}^0 = k_0$) and thus according to BV theory, the following equations are applicable:

$$k_{red} = k_{red}^0 \exp(-\alpha n f \eta) = k_0 \exp(-\alpha n f \eta) \quad \text{Equation 15}$$

and

$$k_{ox} = k_{ox}^0 \exp((1 - \alpha) n f \eta) = k_0 \exp((1 - \alpha) n f \eta) \quad \text{Equation 16}$$

where $f = F/RT$, n = number of electrons involved and α ($0 \leq \alpha \leq 1$) is the transfer coefficient reflecting the symmetry of the barrier to electron transfer and is typically assumed to be 0.5.¹²²

Thus, Equation 14 becomes the following after incorporating the Butler-Volmer equations:

$$i = AFnk_0(\exp(-\alpha n f \eta) \Gamma_{red} - \exp((1 - \alpha) n f \eta) \Gamma_{ox}) = i_0(\exp(-\alpha n f \eta) \Gamma_{red} - \exp((1 - \alpha) n f \eta) \Gamma_{ox})$$

Equation 17

where $i_0 = AFnk_0$. The constant derived in Equation 17, i_0 , is an important parameter in determining the kinetics of an electrochemical system. While the concentration effects determine the thermodynamics of the system, this i_0 value signifies the microscopic flux crossing the interface equally in either directions at equilibrium, *i.e.*, the kinetics of the system. It is not possible to determine i_0 from PFE measurements since the net current at zero-overpotential is always zero.

Marcus-Hush -Chidsey (MHC) theory:¹²⁴ Marcus theory in this thesis refers to the Marcus-Hush -Chidsey (MHC) theory which provides a more accurate description of electron transfer than Butler-Volmer.^{9, 125} Marcus and Hush initially and separately developed the means for calculating outer-sphere electron transfer rates while Chidsey extended it to incorporate interfacial electron transfers by considering the metal-electrode system as an electron donor/acceptor system with a continuum of energy levels.

The following expression is taken from a review by Henstridge *et al*¹²⁴ to denote expressions for the rate constants k_{red} and k_{ox}

$$k_{red} = k_0 e^{-(\frac{1}{2})\theta} \int_{-\alpha}^{\alpha} \frac{\exp[-(\varepsilon-\theta)^2]}{2\cosh[\varepsilon/2]} d\varepsilon \quad \text{Equation 18}$$

$$k_{ox} = k_0 e^{+(\frac{1}{2})\theta} \int_{-\alpha}^{\alpha} \frac{\exp[-(\varepsilon-\theta)^2]}{2\cosh[\varepsilon/2]} d\varepsilon \quad \text{Equation 19}$$

where $\Lambda = \frac{F}{RT}\lambda$, $\theta = \frac{F}{RT}\eta$ and ε is the variable for integration. k_0 denotes the standard electron exchange rate constant as in the case of BV theory. The above equations not only relate the rate of electron transfer to the applied overpotential, η , but also take into account the reorganization energy, λ , that may be involved (Section 1.1).

The two theories are analogous to one another when η is smaller than the reorganization energy, λ but for values of η greater than λ , the Marcus theory becomes relevant in explaining transfer kinetics at the electrode. Were the electron transfer rate the rate determining step in electrocatalysis, the turnover frequency can be determined from either the BV or the MHC theory.

Model by Leger *et al* for residual slope at high potentials: As the overpotential applied to a particular system increases so too does the rate constant until the potential-independent steps of the mechanism become rate-determining and a plateau should be reached in the current response in a CV. In practice, however, the catalytic profile shows a residual slope. This phenomenon was studied by Leger *et al* in 2002 and a model was developed.¹²³ The residual slope was attributed to the inhomogeneous nature of orientations of the enzyme molecules which are adsorbed onto the electrode surface. The various orientations cause dispersion of the interfacial electron-transfer rates with potential and with higher overpotential, those with unfavourable orientations begin to

contribute to the catalytic current resulting in a slope as opposed to a plateau. It may be stated here that the same residual slope is observed for cases when the rate constants for electron transfer are extremely high such that potential-independent chemical steps of the mechanism *does not* become rate-determining.

Turnover signals are best understood by considering the catalytic electron flow through the three-electrode electrochemical set-up as occurring through a series of resistors, namely, R1 , R2 and R3 as shown in Figure 20. The currents which are observed across the various resistances are thus, i_e , i_{cat} and i_{trans} respectively: i_e stands for the current flowing across the resistance offered by interfacial electron transfer across the electrode, i_{cat} reflects the current flowing through the enzyme which offer resistance during catalysis and i_{trans} is the current flowing through the resistance experienced as a result of mass transfer limitations of substrates and products. At an applied potential, V, the observed current, i_{obs} , may be related to the current flowing across the different resistances as

$$\frac{1}{i_{obs}} = \frac{1}{i_e} + \frac{1}{i_{cat}} + \frac{1}{i_{trans}} \quad \text{Equation 20}$$

Ideally, the observed current should reflect the inherent properties of the enzyme (i_{cat}). Generally, interfacial electron transfer is only rate-limiting when enzymes have exceptionally high catalytic current or there is exceptional poor electrical contact between the electrode and the enzyme. To ensure that mass transfer is not rate-limiting, the current is investigated as a function of the rotation rate of the electrode : we need to evaluate the current as per Koutecky-Levich equation (Appendix 2). At adequately fast rotation rates of the electrode, mass transfer is not limited and the ability to avail such a situation is one of the advantages of PFE as an analytical technique.

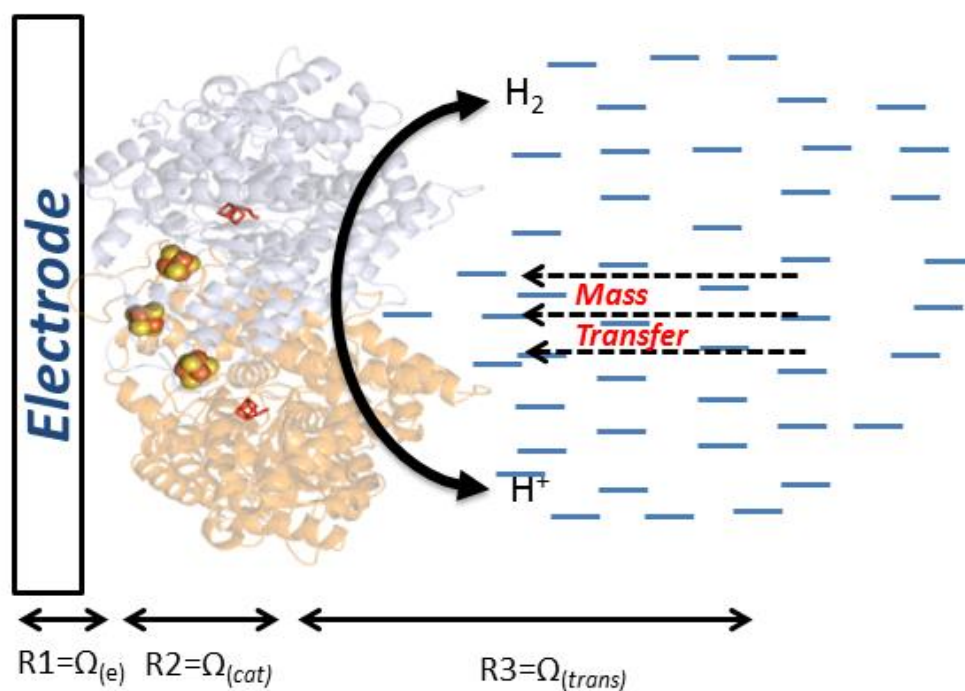


Figure 20: The electrochemical set-up as a series of resistances. The set-up may be considered as a series of three resistances, namely, $R1$, $R2$ and $R3$ through which electron flow occurs during catalysis. The first resistance, $R1$, is that which arises from interfacial electron transfer from electrode to the adsorbed enzyme on it while $R2$ reflects that which arises from electron transfer through the enzyme. $R3$ is the resistance that arises from limitations of mass transfer of substrates from the bulk of the electrolytic solution to the enzyme. In PFE experiments, $R3$ is rendered negligible by rotating the electrode at high speeds (Appendix 2).

2.1.2.3 Non-Turnover signals

The total catalytic current is composed of two parts, namely, faradaic and non-faradaic currents. The Faradaic current can arise from two sources: from turnover (*i.e.* catalytic) or from non-turnover (non-catalytic) states of the system. The catalytic system which results in turnover signals was discussed in Section 2.1.2.2 while non-turnover signals and derivation of electroactive coverage from them are discussed in Appendix 1.

2.1.3 Basis for PFE measurements: Chronoamperometry (CA)

In chronoamperometry experiments, the potential is held constant while the current is recorded. Theoretically, with constant turnover during enzyme-catalysis, the recorded current is constant. However, in practice, a decrease in current is observed over time. This is known as film loss and the extent to which it occurs varies from one experimental run to another. Film loss is attributed to dissociation of the enzyme film from the electrode surface and in a chronoamperometry experiment, it is relevant that the loss is taken into account and corrected for such that the value of current (i) used in quantitative experiments is that which would have existed without the film loss having occurred (Appendix 3). Generally, chronoamperometry is utilized to understand the effect of addition or removal of inhibitors or substrates, temperature and pH changes on the enzyme's activity. Figure 21 shows the utilization of chronoamperometry for the measurement of rate upon addition (Figure 21A) and removal of inhibitor (Figure 21B).

During addition of inhibitors, either as gas into the headspace or as a saturated solution, a drop in current is observed whose initial rate may be assumed to reflect the (relative) rate of inhibition. Recovery from gaseous inhibitors is achieved by a constant flow of substrate or inert gas, the time needed for its removal is dependent on the volume of buffer and the rate of flow of gas. Non-volatile inhibitors or substrates are removed by buffer exchanges.⁴⁴

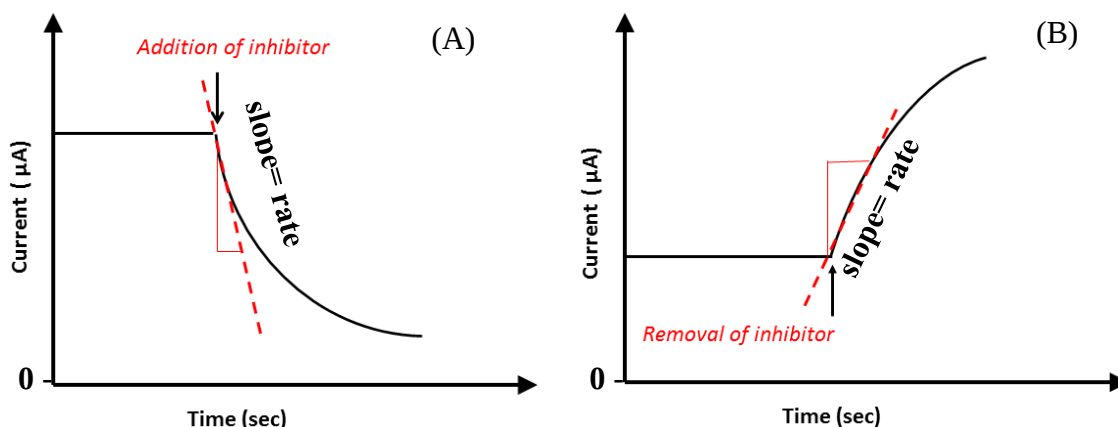


Figure 21: Rates of inhibitions from chronoamperometry experiments. (A) A case of anodic current is shown whose initial values are shown to be constant before an inhibitor is introduced into the system. The current drops upon addition of the inhibitor - the slope of the drop in the initial instance (in red) gives the (relative) rate of inhibition. (B) A case of inhibited system is shown where the current recovers to higher values upon removal of inhibitor, the initial (relative) rate of recovery is given by the slope (in red) as shown. The term 'relative' is used as it is impossible to know the exact turnover when the electroactive coverage, Γ , is unknown during catalysis in PFE experiments.

2.1.4 Applications– to– date of PFE (relevant to the thesis)

The utilization of PFE has been extensive over the past few years: many reactivity characteristics can be measured over a wide potential range by PFE. Usually, slow scans are among the initial experiments carried out on enzymes on a PGE electrode. It allows the following features to be observed: overpotential requirement in either oxidation or reduction reaction, inactivation at high potential and recovery upon reversing potential from that high potential.

Recently, mutations in Hyd-1 around the active site^{44, 126} have successfully been made according to a protocol pioneered by *Hamilton et al.*¹²⁷ In PFE studies on these variant enzymes, one of the characteristics determined was the Michaelis-Menton constant, K_M , which is the substrate concentration required for the turnover rate of the enzyme to

reach half of its maximum value. Usually, it can be approximated as an enzyme's affinity for a substrate, provided that the rate constant for the reverse reaction to enzyme-substrate complex formation is much smaller than the forward reaction of conversion of the enzyme-substrate complex to the enzyme-product complex. The K_M values may be obtained from PFE through a Hanes-Woolf analysis (Appendix 4), which plots $\frac{[S]}{k}$ vs $[S]$ where $[S]$ is concentration of substrate, S, in mol dm^{-3} . In lieu of rate constant, k , the value of the current (i) is measured and recorded since it is directly proportional to k as was given by Equation 2 and reproduced here:

$$i_{cat} = A\Gamma F n k_{cat}$$

where A = area of the electrode surface, Γ = electroactive coverage by enzyme, F = Faraday's constant and n = number of electrons involved in the reaction.

If S is gaseous, then the % composition of S in the gas flow in the headspace is converted to $[S]$ using Henry's law, given in the following equation:

$$c_g = k_H p_g \quad \text{Equation 21}$$

where c_g is the concentration of dissolved gas, p_g is its partial pressure and k_H is Henry's Law constant. As chronoamperometry over a considerable amount of time is usually involved in PFE experiments determining K_M , film loss correction needs to be carried out before analysis (Appendix 3).

Since K_M is potential-dependent, it is important that any experiments carried out to make comparisons between different enzymes are conducted at the same potential and that results are quoted for potential as well as temperature. Furthermore, as K_M is a kinetic parameter, it is important to ensure that mass transport is not a limiting factor. Thus, experiments must be conducted to yield Koutecky-Levich plots at the lowest $[S]$

(Appendix 2) to determine the optimal electrode rotation rate to be subsequently utilized in the K_M experiment.

Another parameter which is measured by PFE is E_{switch} , a parameter defined as the potential of maximum slope in the reductive activation direction.³⁰ Some enzymes have a redox state which is oxidized but inactive and they are often converted to the oxidized active state by reduction : the process is called reductive activation. This conversion is detected in PFE by means of slow scans from CVs which sweep from the positive potential end to the most negative potential end, the potential at which there is maximum slope of the CV is identified as E_{switch} . Simply put, E_{switch} may be thought of as the potential at which two potential-dependent states interconvert. However, through the use of spectroelectrochemical Fourier Transform Infrared Spectroscopy (FTIR) titrations, there is evidence that the E_{switch} measured for hydrogenases value is not the reduction potential of the oxidized inactive state.¹²⁸ A study by Leger *et al* stated that the rates of reactivation may be affected by the reduction potential of the oxidized state to the reduced state, but could also come from the electronic relay system (Fe–S clusters) that connect the active site to the electrode.¹²⁹ Whatever the reason, E_{switch} can conclusively be used to compare the relative “ease” at which an enzyme is reactivated, provided the scan rate is identical between comparisons.¹²⁸

Since oxygen tolerance has been linked to the Fe–S clusters in Hyd-1^{28-29, 34a}, this aspect is explored in this thesis when investigating one of its variants. Comparable levels of oxygen tolerance in both the WT enzyme and the variant would be a good indication that the Fe–S network is intact. Experiments testing exposure to transient oxygen may be performed according to principles outlined in Evans *et al*^{34a} where the

tolerance is investigated *via* PFE using injections of oxygen-saturated buffer. A slow scan rate is deliberately chosen in order to make the injection at a specific potential easier and to allow the gas flow sufficient time to flush the injected oxygen out of the system before the full CV is recorded from a high potential to a lower potential. This ensures that any changes in current are due to the after effects of oxygen exposure rather than oxygen present in solution at that time. To understand inhibition by prolonged exposure to O₂, the catalytic activity can be monitored by chronoamperometry as the % of O₂ in the headspace gas flow is varied from low values to higher values. The % of O₂ may be converted to [O₂], using Henry's law as given in Equation 21. As with the K_M experiment, film loss correction (Appendix 3) needs to be carried out before analysis.

Leroux *et al*¹³⁰ showed that CO and O₂ have the same rate of diffusion into the active site of oxygen tolerant [Ni-Fe] hydrogenases¹³⁰ though they are less sensitive to CO inhibition than they are to O₂.¹⁹ To understand inhibition by CO during prolonged exposure, the catalytic activity is monitored by chronoamperometry with varied percentages of CO in the headspace gas flow. Competitive inhibition between substrate, S, and CO may be modelled by Equation 22 as given below:¹³¹

$$i = \frac{i_{max}}{1 + \frac{K_M^S}{[S]} \left(1 + \frac{[CO]}{K_i} \right)} \quad \text{Equation 22}$$

where i_{max} is the current recorded under 100% S, i is the steady-state current at each CO concentration, K_M^S is the Michaelis-Menton constant for substrate S, K_i is the inhibitor dissociation constant and [S] and [CO] are the concentrations of each species in the cell solution. Plots of $\frac{i_{max}}{i}$ vs [CO] should give a straight line with the slope

providing an indication of CO sensitivity: a *steeper* slope indicates increased sensitivity to CO.

Additionally, activation energy, enthalpy and entropy (E_a , $\Delta H^\ddagger$ and $\Delta S^\ddagger$), kinetic isotope effects and pH optima of enzymes have been measured recently by PFE proving the technique to be an extremely versatile analytical tool.^{126a}

2.1.5 Basis of EIS Measurements: Sequence of Steps

The underlying principle of Electrochemical Impedance Spectroscopy (EIS) technique is the analysis of the current response obtained by application of a small alternating excitation signal (AC voltage) to a sample or electrochemical set-up. Capacitors and inductors introduce time-dependent resistances when AC voltage is applied to a system and the sum total of time-dependent and independent resistances is known as impedance. The impedance response is calculated from the sinusoidal current response which always has the same frequency as the applied voltage but is shifted by phase $\emptyset$ as discussed in Section 2.1. Once these measurements are taken, it is essential to: 1. study the system and the various theories involved in the biophysical processes and 2. create an equivalent circuit which is composed of a virtual arrangement of resistors, capacitors or inductors which mimic the voltage-current relationship seen by the system under study. Steps 1 and 2 are intricately related (as shown in Figure 22) and it is essential that the virtual arrangement not only agree with the observations made but are based on the biophysical processes that may be involved. The experimental results are then fitted the mathematical model corresponding to the virtual arrangement. If the fitting does not align, it proves necessary to revisit the understanding of the system or the virtual arrangement. A fitting allows numerical values to be obtained for the

components of the arrangement and hence to the ‘resistor-like’, ‘capacitance-like’ and ‘inductor-like’ components of the system under study.

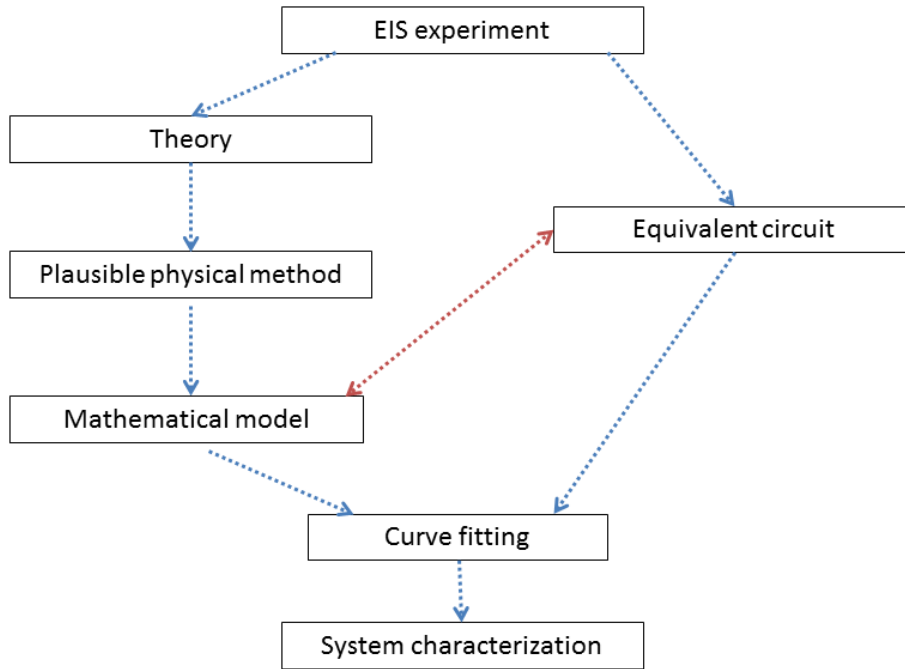


Figure 22: The steps involved in EIS analysis. The sequence of steps that need to occur before system characterization can be done with EIS are development of an equivalent circuit based on the biophysical processes of the system and subsequent development of a mathematical model to which the experimental results are fitted. An fitting of the experimental results to the model allows the assignment of numerical values to the ‘resistor-like’, ‘capacitance-like’ and ‘inductor-like’ components of the system under study.

2.1.5.1 Nyquist and Bode Plots

During the measurements, an analyser tracks the sinusoidal current response obtained by application of a sinusoidal voltage; the voltage and current sinusoid signals are always at the same frequency but are shifted in phase. As covered in Section 2.1, impedance, Z , is given by Equation 8. It is reproduced below as

$$Z(t) = \frac{V(t)}{I(t)} = \frac{V_0 \cos(\omega t)}{I_0 \cos(\omega t - \phi)} = \frac{V_0 \exp[j(\omega t)]}{I_0 \exp[j(\omega t - \phi)]} = Z_0 \exp[j\phi]$$

which is not constrained by any time-domains.

By applying Euler's relationship to Equation 8 and replacing Z_0 with $|Z|$, we get

$$Z = Z_0 \exp[j\emptyset] = |Z| (\cos\emptyset + j\sin\emptyset) \quad \text{Equation 23}$$

where the magnitude $|Z|$ is given by

$$|Z| = \sqrt{(Z')^2 + (Z'')^2}$$

Equation 23 may be expressed in another manner as given below:

$$Z = Z' + jZ'' \quad \text{Equation 24}$$

where, $Z' = |Z|\cos\emptyset$ and $Z'' = |Z|\sin\emptyset$ and are the real and the imaginary parts of the impedance, respectively. The real and imaginary parts of the impedance are related to the phase angle $\emptyset$ by

$$(\emptyset) = \tan^{-1}\left(\frac{Z''}{Z'}\right) \quad \text{Equation 25}$$

and may be plotted in a complex plane. In the complex plane, Z'' is plotted against Z' to yield a Nyquist plot with $(Z', -Z'')$ forming just one point on the plot. However, EIS measurements can be taken at various frequencies to yield a trace of $(Z', -Z'')$ points on the Nyquist plot and it is this trace which indicates the type and arrangement of components to include in the virtual circuit. Alternatively, the result of an EIS measurements can be made as a Bode plot in which both $\log |Z|$ and $\emptyset$ are plotted against $\log \omega$. Sometimes, it is helpful to plot $\log Z''$ against $\log \omega$.¹³²

2.1.5.2 Modelling of circuit

Nyquist and Bode plots allow the analysis of any electrochemical system by considering it as a circuit (an assembly) of three circuit components: resistance, capacitance and inductance. The resistance (R) is given by the real portion of Equation 23/ Equation 24 while capacitance (C) and inductance (L) both contribute to the imaginary portion.

One of the most common arrangements encountered in electrochemical systems is that of a resistor and a capacitor placed in parallel.³⁵ This combination is given a special name: it is called a **RC element** and is shown in Figure 23 along with its Nyquist plot. Such an arrangement of components yields a plot with a semicircular shape as shown in Figure 23A. Left-hand side of the arc is made up of points measured at higher frequencies while those at the right-hand side are of lower frequencies. A Nyquist plot with the arc having a slight offset along the x-axis would indicate the presence of a resistor in series of the RC element as the x-axis represents pure resistances (Figure 23B). In electrochemical systems, this resistance is often the solution resistance, R_s . It is the combination of all the resistances offered by leads and connections that lead to the electrochemical cell as well as the bulk of the solution within the electrochemical cell.

While the left-hand side of the arc represents a combined resistance of R and R_s , the right-hand intercept of the arc represents an additional resistance R_c , the resistance offered by the double-layer that forms at the electrode. The double-layer has a capacitive nature but, at the lower frequency of the right-hand intercept, the double-layer is fully charged and exerts a resistance which is represented by the real portion of the impedance measurement. Any other point on the arc represents the impedance manifested by an incompletely charged double layer and the R and R_s elements.

The solution resistance, R_s , in series with the RC element forms what is known as the Randles circuit. While R_s and R are related to the real portions of Equation 23/ Equation 24, the impedance displayed by any capacitance, Z_C , is related to the imaginary portion by:¹³³

$$Z_C = \frac{1}{j\omega C} \quad \text{Equation 26}$$

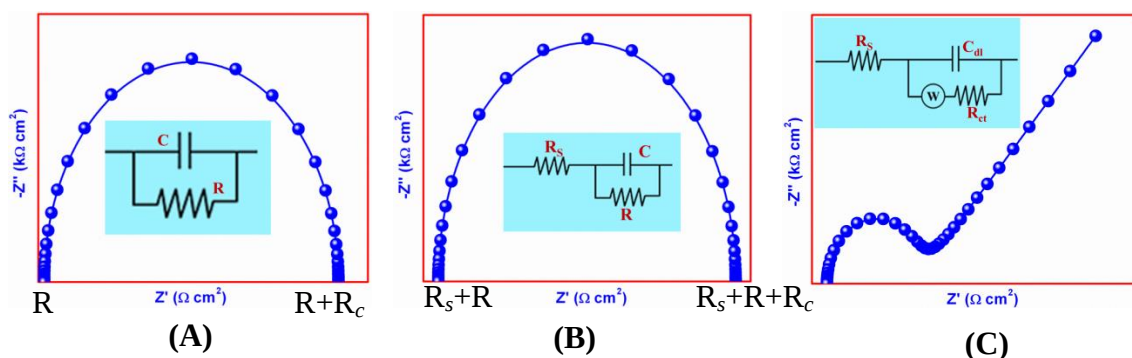


Figure 23: The circuit with the resistance (R) and capacitor (C) components in parallel along with its Nyquist plot. (A) The most common arrangement of virtual electrical components that mimic electrochemical systems in impedance measurements is the resistance (R) and capacitance (C) placed in parallel in a circuit. The corresponding Nyquist plot traces an semicircular arc which cut the x-axis (the real axis) at numerical value of R. Each of the blue spheres represent a $(Z', -Z'')$ point obtained at a certain frequency and a culmination of all the measurements over a range of frequencies give the arc trace. The intercept on the right-hand side correspond to the combined resistance of R and C which is fully charged. (B) The Randles circuit consists of the same parallel RC circuit with a solution resistance, R_s , in series with it. The arc is the same as shown in (A) but is displayed by a value corresponding to R_s . The intercept on the right-hand side correspond to the combined resistance of C which is fully charged, R and R_s . Any other point on the arc contains a contribution of the imaginary portion by C and the real portion by R to the total impedance of the system. (C) The Randles circuit shown in Panel B now incorporates a Warburg element (W) which represents the impedance manifested by semi-infinite diffusion by electroactive species. A straight line at 45° angle appears after an incomplete semicircular arc. The figures were kindly provided by Dr. Kavita Pandey.

In an electrochemical system, although the pure capacitance is displayed as a perpendicular straight line to the x-axis (the Z' axis), an angle lower than 90° may sometimes be observed in the complex plane plot. Such behaviour is attributed to (i) microscopic roughness caused by scratches, pits, etc. always present on solid surfaces and (ii) a capacitance dispersion of interfacial origin connected with slow adsorption of ions and chemical inhomogeneities of the surface. In equivalent circuit model of an electrochemical system, the capacitance is thus often replaced by a constant

phase element (CPE) to account for the deviation from an ideal capacitor. This is shown in the circuit diagram (Figure 22C) as C_{dl} .

The impedance of the CPE is expressed as:¹³³

$$Z = \frac{1}{(j\omega)^n Q_n} \quad \text{Equation 27}$$

where, Q_n and n are the CPE pre-factor and index, respectively. If the index n is equal to 1.0 the CPE coincides with a pure capacitor. Generally, n varies from 1.0 to 0.5 to fit an experimental data. Q_n is equal to the reciprocal of the impedance when the frequency, ω , is equal to one.

Another component which is often found in electrochemistry is the impedance manifested by electroactive species in the bulk of the solution and known as the Warburg element (W). Its semi-infinite diffusion is modelled by the diffusion component, Z_W , and can be expressed as:¹³⁴

$$Z_W = \sqrt{\frac{2\sigma}{j\omega}}$$

Since $\frac{1}{\sqrt{j}} = \frac{(1-j)}{\sqrt{2}}$, the above may be written as

$$Z_W = \frac{\sigma (1-j)}{\sqrt{\omega}}$$

The coefficient σ is known as the Warburg coefficient and is dependent on the bulk concentration and diffusion coefficient of the oxidant and reductant species, the surface area of the electrode and the number of electrons involved. This results in the formation a straight line at 45° angle to appear after an incomplete semicircular arc (Figure 23C).

Thus, a Randles circuit with CPE instead of a pure capacitor and incorporating a Warburg element is given by the relationship:

$$\text{Impedance, } Z_{\text{circuit}} = R_s + Z_{RC \text{ element}}$$

$$Z_{\text{circuit}} = R_s + \frac{R_{ct} + Z_w}{1 + \frac{1}{(j\omega)^n Q_n} (R_{ct} + Z_w)} \quad \text{Equation 28}$$

2.2 Relevant Molecular Biology Theory: Site Directed Mutagenesis (SDM)

The process of replacing one residue with another in the polypeptide chain by targeting the gene sequence is called site-directed mutagenesis (SDM). It is a technique which is used to introduce specific amino acid changes in an expressed gene product through the introduction of base pair changes at defined positions within the relevant gene.¹³⁵ SDM is especially useful for elucidating structure –function relationships in enzymes and other proteins; substituting one residue by another allows the inference of the role of the former residue by studying the changes in performance of the new structure.

When considering suitable methodology for the mutagenesis, thought must be given as to how to introduce a mutation without disrupting the biosynthesis of the enzyme. The following sections involve detailing SDM of Hyd-1 from *E. coli*, so the first consideration was the biosynthesis of *E.coli* Hyd-1 which involves a multi-step pathway with multiple accessory proteins: the enzyme is coded for by *hyaA* and *hyaB* genes of the *hyaABCDE* operon (Figure 24).¹³⁶ Given this complexity, a route where the protein is expressed from the wild-type (WT)/native organism and the expression levels of all accessory proteins are maintained at WT levels is preferable to a route involving over-expression.

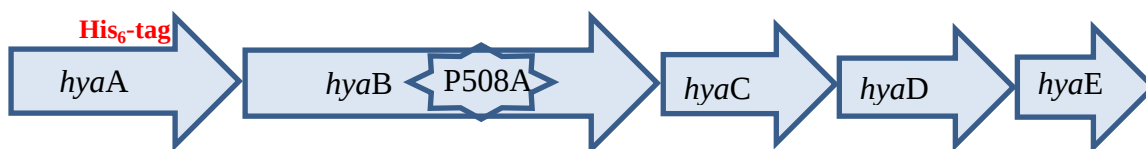


Figure 24: The location in the genetic sequence at which the P508A mutation is to be carried out. The formation of hydrogenase-1 from *E. coli* involves the coordinated activities of various genes. The *hyaB* gene is where the intended mutation is to take place in order to affect the large subunit where the active Ni-Fe active site is located. The *hyaA* gene of the cell strain used for site-directed mutagenesis (SDM) contains a polyhistidine tag at the C-terminus which enables enzyme purification by Ni-affinity chromatography.

All mutation studies involving *E. coli* Hyd-1 carried out previously in the FAA group were from encoding the mutation into the chromosome in order to avoid issues arising from the complicated regulation and maturation of these enzymes.^{19, 34a, 44} A bacterial strain (made by Frank Sargent and Tracy Palmer from the University of Dundee) with an additional polyhistidine tag to the sequence of *E. coli* Hyd-1 is the MC4100-derived FTH004 cell strain. The FTH004 cell strain was utilized in the SDM work leading to this thesis as the hexa-histidine tag (located at the C-terminus of the hydrogenase small subunit, HyaA) enables purification using nickel-affinity chromatography.¹³⁷

2.2.1 pMAK 705 plasmid as vector and template

Introduction of the mutation directly onto the chromosome of the WT organism is not always possible, especially, if it is large in size.¹³⁸ A satisfactory route is the use of a vector to which the required changes may be applied and which will then transfer these mutation(s) to the chromosome; pMAK 705 is a plasmid that is suited to this function. It is readily inserted into and replicated in competent DH5 α cells— ‘competent’ is a term denoting being capable of DNA uptake (Appendix 5). The DH5 α cells are *E. coli*-derived cells available commercially for general cloning and sub-cloning purposes.

They possess high transformation efficiency, that is, they have the ability to form a high number of colony forming unit ($>10^9$ cfu/ μ l) and are thus suited to serve as the vehicle from which stocks of mutated plasmids may be formed. The transformation is more commonly achieved by heat shock treatment (Appendix 6) though electric pulses may be used. Exposure to heat (at 42°C for 40s) or electric pulses for a millisecond duration open up pores on the cell membrane to allow the free DNA into the cells.

The *pMAK 705* plasmid vector is characterized by its small size with only 5593 base pairs (bp). This work utilized *pMAK 705_hyaB* plasmid which had previously been modified to contain the *hyaA*, *hyaB* and *hyaC* genes into it (8392bp).^{19, 34a, 44, 139} It has a crucial gene conferring chloramphenicol (Cam) resistance, Cam^r, and a temperature-sensitive pSC101 origin of replication, Rep^{Ts}, which enables replication of the free plasmid at 30 °C but not at 44 °C. These two features allow *pMAK 705_hyaB* to be an efficient vector as it allows for subsequent screening for cells with desired mutation based on Cam resistance and growth temperature: the protocol for screening and insertion of desired mutation into the chromosome was designed by Hamilton *et al*¹²⁷ which is described in Section 2.2.3. Generally, a codon change of one residue is carried out at a time (1-3bp).

2.2.2 Gibson Assembly Procedure

Generally, mutations can be introduced into the template directly by use of suitable oligonucleotide primers which overlap in the regions of interest. However, a method pioneered by Gibson *et al*¹⁴⁰ and known as the Gibson assembly procedure was utilized for the SDM procedure in our work. This method utilizes an initial polymerase chain

reaction (PCR) step to create double stranded (*ds*) linear DNA with mutation and which are then ligated to yield the circular plasmid DNA (in this case, *pMAK 705_hyaB*).

Primers for PCR, compliments to the mutagenic primers, are deliberately chosen so as to avoid the *pSC101* (Rep^{Ts}) and Cam^r cassettes, reducing the risk of introducing mutations into these key features. The reaction products from the PCR reaction are then subjected to a *DpnI* digestion in order to destroy any template DNA. *DpnI* is an exonuclease enzyme that targets and cleaves DNA at the restriction site of methylated adenine of a sequence which occurs only in naturally occurring DNA and does not affect DNA made by PCR.

The linear sequences made by PCR (Figure 25A) can be separated by agarose gel electrophoresis and then subjected to an enzymatic assembly procedure *in vitro* to ligate the strands of *ds*-DNA together into one molecule (Figure 25B). Three enzymes are used: a DNA polymerase, 5'-exonuclease and ligase. The exonuclease does not compete with polymerase activity making all three enzymes suitable for use in a single isothermal reaction and chews back the *ds*-DNA fragments from their 5' ends to create single-stranded 3' overhangs that are complementary and can anneal. After that, the DNA polymerase extends the 3' ends of the *ds*-DNA to fill in any gaps within each annealed fragment before the ligase seals any gaps that remain.

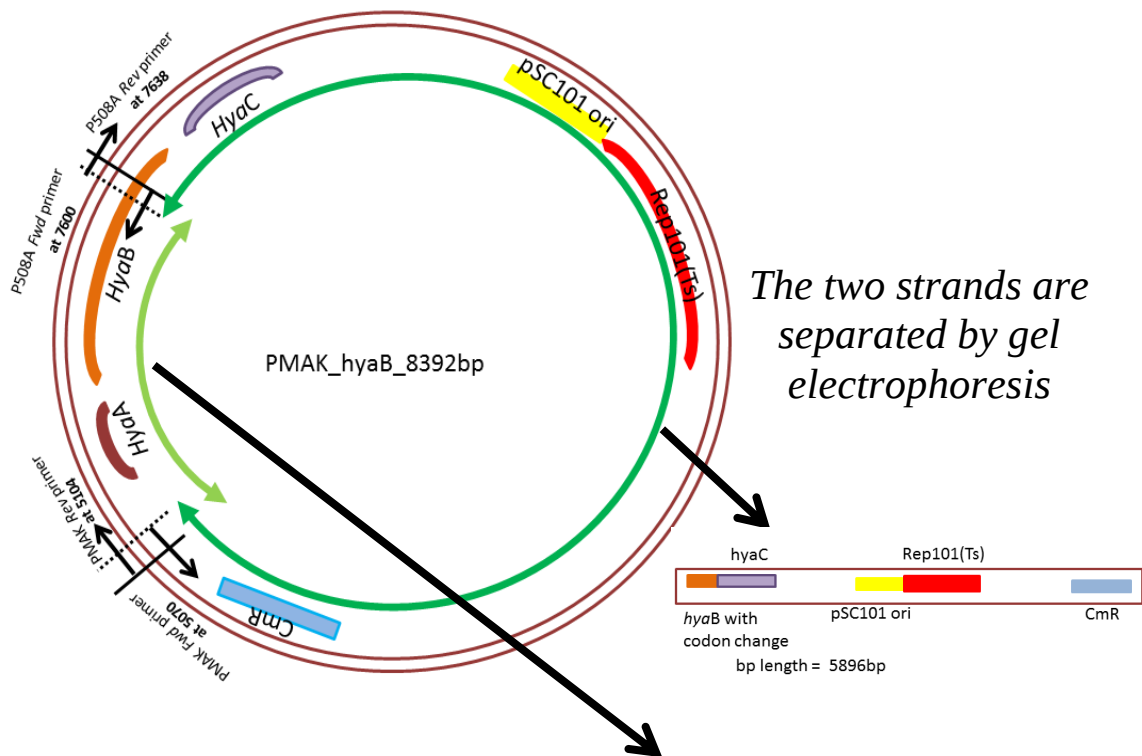
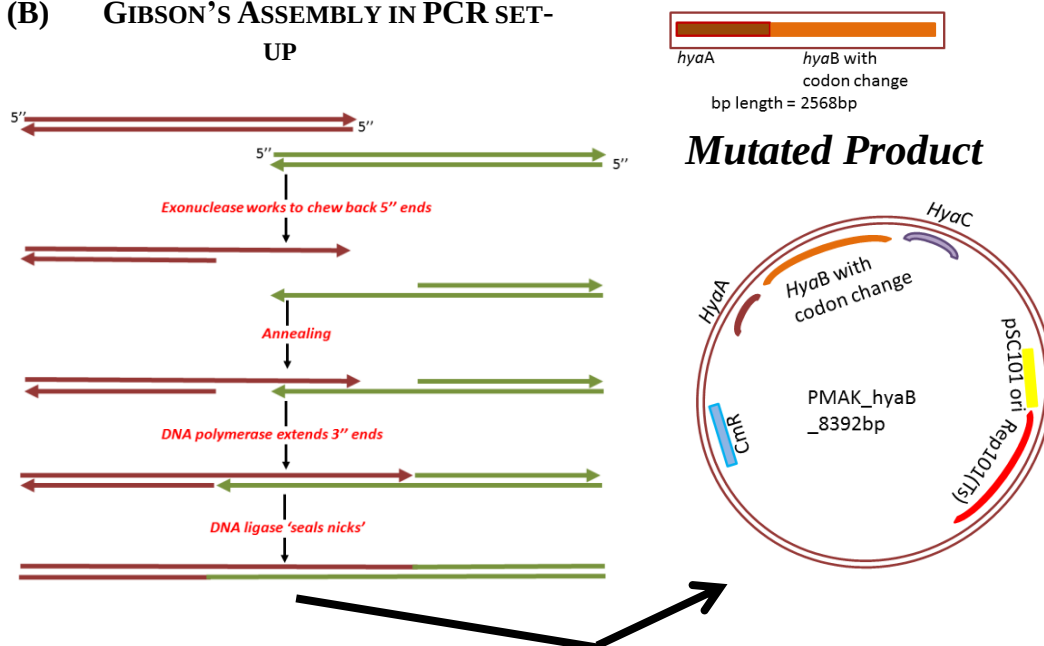
(A) INITIAL PCR REACTION YIELDS LINEAR STRANDS**(B) GIBSON'S ASSEMBLY IN PCR SET-UP**

Figure 25: Codon change brought about by Gibson Assembly procedure¹⁴⁰ which is carried out in two parts: initial PCR and Gibson Assembly. (A) The initial PCR generates two strands which are linear and which can be seen and collected as bands by agarose electrophoresis by matching to their calculated sizes (5896bp and 2568bp). (B) The Gibson Assembly portion utilizes three enzymes: a DNA polymerase, 5'-exonuclease and ligase to yield the circular 'scarless' plasmid with mutation on it.

2.2.3 pMAK Protocol for Introducing Mutations into Chromosomes

The pMAK protocol, as developed by Hamilton *et al*, is a methodology used to introduce gene replacements within *E. coli* chromosomes.¹²⁷ The pMAK plasmid contains two notable cassettes, a temperature sensitive origin of replication, pSC101 (Rep^{Ts}), which allows for plasmid replication at 30 °C but not 44 °C, and Cam^r, a gene cassette that confers resistance to chloramphenicol (Cam).

A gene sequence, homologous to the chromosomal DNA but containing the desired mutation, is inserted into a vector plasmid and an initial recombination event occurs between the gene on the chromosome. This leads to the formation of a **co-integrate** which can be selected for *via* its Cam resistance and growth at 44 °C (the non-permissive temperature of replication of the plasmid) since any cells still containing free plasmid will not grow (Figure 26).

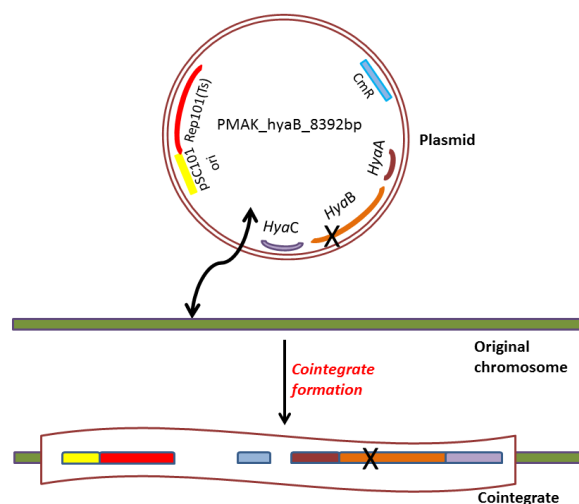


Figure 26: Formation of co-integrate *via* homologous recombination. Adapted from Hamilton *et al*.¹²⁷ The green bar represents the original gene on the organism's chromosome while the brown circular plasmid contains the mutation as noted by a 'X'. The black double-headed line shows the crossover event that leads to homologous recombination that leads to the formation of the required co-integrate. The formation of a co-integrate can be confirmed and selected for *via* its Cam resistance and growth at 44 °C (the non-permissive temperature of replication of the plasmid) since any cells still containing free plasmid will not grow at that temperature.

After selection, the cointegrates are grown at 30 °C (the permissive replicative temperature of the plasmid) leading to a second homologous recombination event which releases the plasmid back into the cell (Figure 27). These cells can then be selected for since growth from Rep^{Ts} occurs faster in the free plasmid than it does on the chromosome.

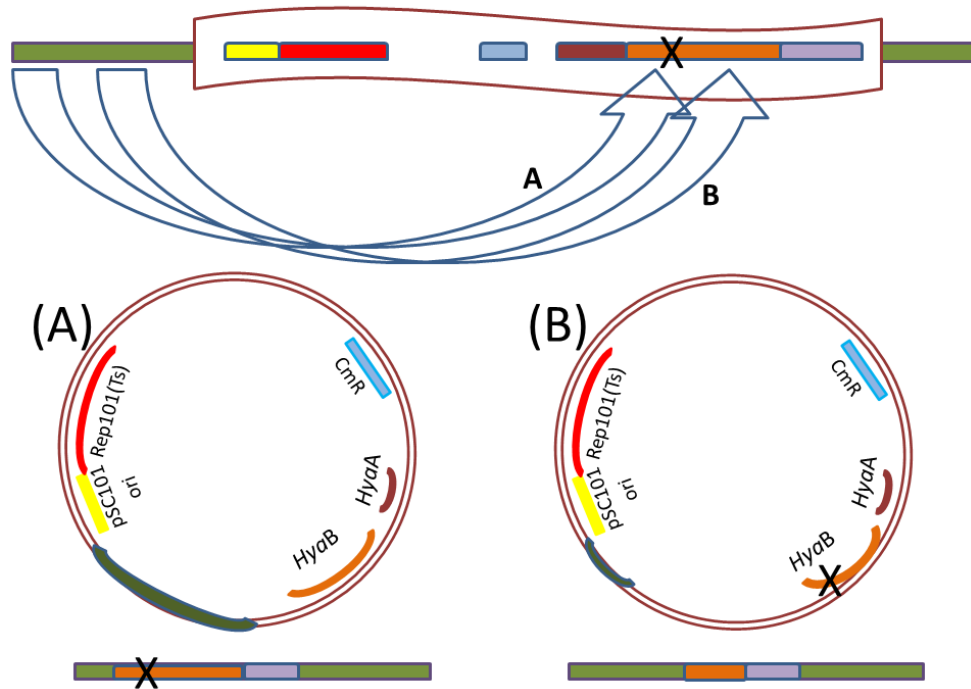


Figure 27: Second homologous recombination to release the plasmid from the cell is shown by arrows labelled A and B. Adapted from Hamilton *et al.*¹²⁷ The plasmid by crossover event A retains mutation in the chromosome while crossover B results in plasmid with mutation. The desired mutant cells (mutation in the chromosome) are selected for by growth as those possessing plasmid with Rep^{Ts} grow faster.

Further growth at 44 °C is used to inhibit plasmid replication and remove the plasmid DNA from the cells. Replica patches are then made on plates with and without Cam in order to confirm the absence of plasmid DNA. The Cam-sensitive (therefore without plasmid), colonies contain the gene in question which may be amplified *via* colony PCR and submitted for sequencing to confirm the presence of the mutation.

2.2.4 Isolation and Purification of enzymes

Unlike many organisms possessing O₂-tolerant enzymes,¹⁴¹ the expression of all *E. coli* hydrogenases is inhibited by O₂.¹⁴² When *E. coli* is grown anaerobically on non-fermentable carbon sources, exponential phase growth is accompanied by expression of Hyd-2 while Hyd-1 is expressed during stationary phase (a low pH and addition of formate are known to upregulate this expression). Also, the *hya* operon responsible for Hyd-1 production is most strongly expressed in the stationary phase of anaerobic growth¹⁴³ although its expression has also been detected in aerobically grown *E. coli*.¹⁴⁴ Thus growth of Hyd-1 *E. coli* is carried out under anaerobic conditions until stationary phase is reached. Whether or not the growth is at exponential or stationary phase can be determined by measuring the optical density of the bacterial culture. The optical density of the bacterial culture samples is obtained by measuring the absorbance of 600 nm light in a quartz cuvette. The absorbance, A, is defined as:

$$A = -\log (I / I_0) \quad \text{Equation 29}$$

where A is the absorbance, I is unscattered light i.e. the transmitted light and I₀ is incident light. The optical density (OD) depends on the pathlength (L) and is defined as OD = A / L.

Hyd-1 is a membrane-bound protein and thus to isolate the protein, the cells from the growth of the bacterial culture are disrupted by French Press to yield membrane-bound samples which are then homogenized and solubilized (Appendix 7).⁵⁷ Purification is carried out (under aerobic conditions) by monitoring UV measurements that occur during the enzyme's passage through a nickel affinity column fitted onto an Äkta purification system. Once immobilized on the column, the enzyme is then eluted by imidazole concentration gradient– purified enzyme is collected from a set of elution

which corresponds to peaks of both 420nm (absorbance by iron (Fe) in the protein) and 280 nm (absorbance by aromatic amino acids tryptophan and tyrosine in protein). The collected enzyme should thus, in all probability, contain proper protein formation with Fe incorporation. Purification of WT Hyd-1 for PFE have been known to incorporate HyaA, HyaB and HyaC units (as per standard lab protocol for PFE experiments (Appendix 7)) and experience within the groups indicate that the presence/absence of HyaC does not alter the electrocatalytic profiles obtained during PFE measurements. The presence of HyaA and HyaB is imperative to getting proper profiles.^{19, 34a, 57} Preparations for forming crystals are subjected to extra steps of size exclusion and hydroxyapatite purification (Appendix 7).

2.2.5 Integrity of enzymes (Solution Assays)

Solution assays are one means of ensuring the integrity of purified enzymes (either WT or its mutated variant). There will be a change in activity between the WT and the variant but the ability to catalyse the reaction it was meant for is indicative of integrity of formation of the enzyme molecules. Another method is crystallization – the formation of the crystals of the protein sample and subsequent modelling allows one to ‘view’ the placement of the residues and to assess the integrity of the enzyme formed. The proper formation of the mutated sample can also be assessed by genetic sequencing of the cells thought to contain the mutation. This three-pronged approach was used in this work for confirming the integrity of the P508A variant of *E. coli* Hyd-1 obtained by SDM. Crystal formation and modelling was carried out by Dr. Stephen Carr (Research Complex at Harwell (RCaH), Didcot, UK) and the P508A mutation of *E. coli* Hyd-1 has been deposited into the Protein Data Bank (PDB) with PDB code: 5JRD. Complete genetic read was carried out by submission of samples to Source Bioscience which

confirmed that the intended mutation (P508A) was incorporated into the HyaB gene on the chromosome.

Solution assays is a means of analysis of steady-state enzyme kinetics and uses visible and UV spectrophotometric methods in which the absorbance during the reaction forms the basis for measuring enzyme activity. Either the substrate or the product of the enzyme-catalysed reaction absorbs a specific wavelength of light. The reaction can then be followed by monitoring the decrease in absorbance by substrate as its concentration decreases throughout the reaction or by monitoring the increase in absorbance by the product as its concentration increases during the course of the reaction. Based on the rate of change of the absorbance units, rate of an enzyme-catalyzed reaction can be expresses as $\mu\text{mol } [S] \text{ min}^{-1} \text{ mg}^{-1} \text{ enzyme}$ where S is the substrate of the reaction.

Nowadays synthetic dyes are used for study of oxidoreductases when neither the substrate nor the product is coloured. Methylen blue (MB) is one such example of what is known as a mediator.¹⁴⁵ This dye changes colour from a deep blue in an oxidizing environment to colourless when in an reducing environment. In the case of following the reaction catalyzed by hydrogenases which are oxidizers, the blue MB solution is saturated with H_2 by bubbling. A measured quantity, usually 1ml, is then transferred to a quartz cell to allow tracking of change of absorbance by a UV spectrophotometer. Minute quantity of the enzyme is then introduced into the cell by Hamilton syringe. As soon as the enzyme is added, the H_2 in the solution is oxidized by it and the electrons from the reaction provide the reducing environment for MB, rendering them colourless. The change in absorbance from conversion from blue to colourless, measured at 600nm, thus corresponds to the rate at which the enzyme can

catalyze the oxidation of H_2 . As mentioned before, the results can be expressed as $\mu\text{mol [S] min}^{-1} \text{mg}^{-1}$ enzyme or as turnover frequency (s^{-1}), provided that it can be ensured that the MB solution had been saturated enough (here, taken as 5 times the concentration of the value of $K_M^{\text{H}_2}$)^{126a}. The protocol adopted for utilizing methylene blue for solution assays for both WT and the P508A variant is given in Section 8.5.5.

Chapter 3: Site-Directed Mutagenesis of Hydrogenase-1

3.0 Abstract

Hydrogenase-1 (Hyd-1) from *Escherichia coli* is a [Ni–Fe] hydrogenase which is an H₂–oxidiser with huge potential for use in envisioned H₂ economy where H₂ can be used as a clean carbon-neutral fuel. Significant research into biomimetics based on the active site of Hyd-1 have been taking place: the active site is made of low-cost and yet abundant transition metals and carries out H₂ oxidation at high turnover rates. Recently, arginine at the 509 position has been proposed as an important residue in H₂ activation: the work in this study concentrates on the structural importance of another residue, proline at the 508 position, and its role in the pre-organization of the aforementioned arginine. This study reports the successful mutation of proline at the 509 position with alanine and an investigation into the impact on reactivity characteristics by protein film electrochemistry (PFE) due to the mutation. It was concluded that while some local instability was introduced upon replacement of proline with an alanine residue, the overall structure remained stable. However, it may be concluded that the pre-organization of the arginine residue brought about by the proline residue is deteriorated upon replacement with alanine through effect(s) on the chemical step(s) of H₂ activation: the effect may, however, be mitigated by other residues as evidenced in nature. The discovery of this provides another piece of knowledge in the quest for gaining the ‘*design principles*’ for H₂– economy enabling biomimetics from Hyd-1 of *E. coli*.

3.1 Introduction

Hydrogenases are metalloenzymes found in organisms belonging to the archaea, bacteria and less commonly, eukarya domains of life.⁵¹ Convergent evolution has led to the formation of three types of hydrogenases, namely, [Ni–Fe], [Fe–Fe] and [Fe] hydrogenases of which [Ni–Fe] hydrogenases are known to be O₂ –tolerant and H₂–oxidizers.^{34a} With immense potential in technological applications, the mechanism of how [Ni–Fe] hydrogenases catalyze H₂ activation is of considerable interest. Since the formation and solving of crystal structures of hydrogenases, there have been many biomimetics made based on the active sites such that H₂ oxidation can be carried out.^{146, 146b, 147} It has been a long-held assumption that during H₂ activation in a [Ni–Fe] hydrogenase, the thiolate sulfur (S) ligands from one of the cysteines that bind the Ni of the bimetallic centre to the protein scaffold act as the base for heterolytic H–H bond cleavage.^{54a} The fact that simple Ni complexes with terminal thiolates are not good catalysts has led to an alternate proposal by Evans *et al*⁴⁴ that a strictly conserved arginine situated <4.5 Å from the bimetallic active centre (residue no. 509 in the sequence as numbered in *E.coli* Hyd–1) serves as the required base while either the Ni or Fe of the active site acts as an acid to supplement a Frustrated Lewis Pair (FLP) system.⁶⁸ The importance of the nitrogen (N) as a potential part of the hydrogen activation and proton transfer in the catalytic cycle of [Fe–Fe] hydrogenases has already been emphasized.^{146a} O’Hagan *et al*¹⁴⁸ reported that the efficiency of one of their catalysts depended on the position of the N–H bond with respect to the Ni; they calculated the distance between the Ni and N in their catalyst to be less than 4.5 Å, a similar distance to that of Ni or Fe in the active site to the R509 residue.⁵⁸

Sequence alignment and study of conserved residues among genetic sequences form the basis for elucidating various structure– functional relationships. Evans *et al*⁴⁴ probed the conservation of residues at 509, 118 and 574 positions of *E. coli* Hyd-1 for investigating their roles in H₂ activation by protein film electrochemistry (PFE). These residues form what was defined by Evans *et al*⁴⁴ as the canopy residues: the canopy region is defined as a collection of conserved residues which forms the outer coordination sphere necessary for the functioning of the bimetallic centre and closest to the site at which exogenous agents CO and O₂ interact and substrate H₂ binds, and a hydrido intermediate is stabilized.

Figure 28A shows a sequence alignment which compares the primary sequence of *E.coli* Hyd–1 with that of other O₂–tolerant [Ni–Fe] hydrogenases (Group 1d of classification as shown in Appendix 10) as well as with some Group 1a [Ni–Fe–Se] hydrogenases (part of subgroup of [Ni–Fe] hydrogenase) that contain serine as opposed to an aspartate in the 118 position (Hyd-1 numbering) and a seleno-cysteine instead of a cysteine in the 576 position (Hyd-1 numbering) holding the Ni to the protein scaffold. A closer look at the alignment reveals that a proline residue at the 508 position (P508) is also conserved across the series (highlighted in yellow in Figure 28A). Figure 28B shows the proline holding the arginine in position above the Ni–Fe bimetallic active site in Hyd–1 (PDB code: 5A4M).^{44, 126b}

A recent paper by Brooke *et al*^{126a} calculated hydrogen activation enthalpy and entropy ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) at the active site of a Hyd–1 variant in which the arginine residue was replaced by a lysine residue by protein film electrochemistry (PFE). It was found that the R509K variant showed very low activity towards hydrogen oxidation although it

possessed a lower value of $\Delta H^\ddagger$. This suggests a less favourable $\Delta S^\ddagger$ as the more dominant factor in determining rates of activity; the lysine residue is unable to maintain the ordered form as in the case of arginine residue. It was assumed that the ability to form salt-bridges with nearby aspartate residues at 118 and 574 were affected. However, the study by Evans *et al*⁴⁴ revealed that the replacement of the aspartate residues with neutral residues did not alter the potentially mobile arginine and gives rise to the hypothesis that the stability of the active site comes from elsewhere. A proline residue holding the arginine could be the source of the pre-organization of the arginine residue.

Proline residues are aliphatic with no functional groups—its uniqueness coming from being covalently bound to the nitrogen of the peptide backbone and the ability to impart rigidity through constraint on rotation of the N–C(α) bond. This means that this residue has no amide hydrogen to take part as donor in hydrogen bonding or resonance stabilization. The P508 residue may be found to be located at the $i+1$ position of a type I beta (β)–turn, a position where proline is typically favoured due to its stabilizing effect on the local protein fold.¹⁴⁹ Beta turns are common when making the tight turn for antiparallel β –sheets and usually involve four residues two of which are non–hydrogen bonding and two which are hydrogen–bonding residues on either side. Proline is one of the non–hydrogen bonding residues. These turns occur where there is access to the solvent but to occur as they do in Hyd–1, near the active site, and for the proline to be positioning the arginine above the bimetallic centre, may prove to be highly relevant.

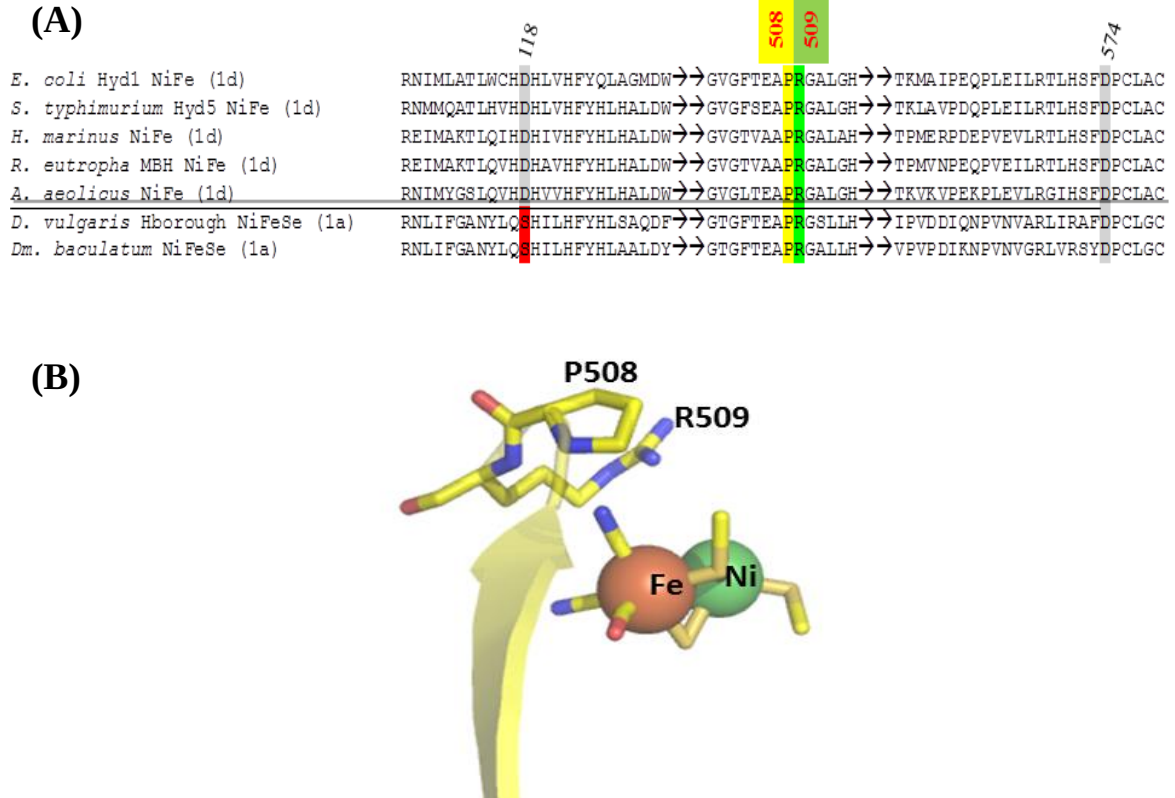


Figure 28: (A) Sequence alignment of canopy region residues. Selected portions of the large subunits of various O_2 -tolerant (Group 1d, top tier) and [Ni-Fe-Se] hydrogenases (Group 1a, bottom tier) are shown. Canopy residues at 118 and 574 are highlighted in grey and the Asp-to-Ser substitution at position 118 is highlighted for the [Ni-Fe-Se] hydrogenases in red. The arginine residue at position 509, considered essential to H_2 activation, is highlighted in green while the adjacent conserved proline at 508 (the subject of this study) is highlighted in yellow. *E. coli* Hyd-1 numbering is used. (B) Crystal structure of the active site of *E. coli* Hyd-1 highlighting proline at the 508 position which holds the arginine at the 509 position in place (PDB code: 5A4M)⁴⁴.

To determine the role of the proline, replacement of the residue was made with alanine which is neutral as proline but capable of imparting flexibility to the arginine residue that is held over the bimetallic centre (by site-directed mutagenesis). Additionally, the replacement with alanine showed *via* Pymol software that the arginine residue would maintain its distance from the bimetallic centre.

3.2 Results of Molecular Biology Procedures

3.2.1 Formation of mutated *pMAK_hyaB_P508A*

As discussed in Section 2.2, it is possible to create a mutated plasmid by use of primers containing the mutation. Gibson Assembly¹⁴⁰ was used to form the mutated plasmid, the initial requirement of which was the formation of *ds*-DNAs of appropriate lengths (Section 2.2 and Section 8.5.1). The confirmation of the lengths come from observing bright bands compared to a 1kb ladder which were then gel extracted (Figure 29). The *ds*-DNAs were then assembled to form the plasmid vector *pMAK_hyaB_P508A* under conditions which are given in Section 8.5.2.

3.2.2 Growth curve of *E. coli* containing the mutation

Competent DH5 α cells were transformed by *pMAK_hyaB_P508A* plasmid by heat shock treatment (Appendix 6) to provide amplification of the plasmid which was then transferred into the chromosome sequence of FTH004 cells¹³⁷ by *pMAK* protocol (Section 2.2.3). The bacterial FTH004 cell strain is MC4100-derived and encodes for *E. coli* Hyd-1 with polyhistag. The hexa-histidine tag is located at the C-terminus of the genetic sequence encoding the hydrogenase small subunit in order to enable purification using nickel-affinity chromatography.¹³⁷

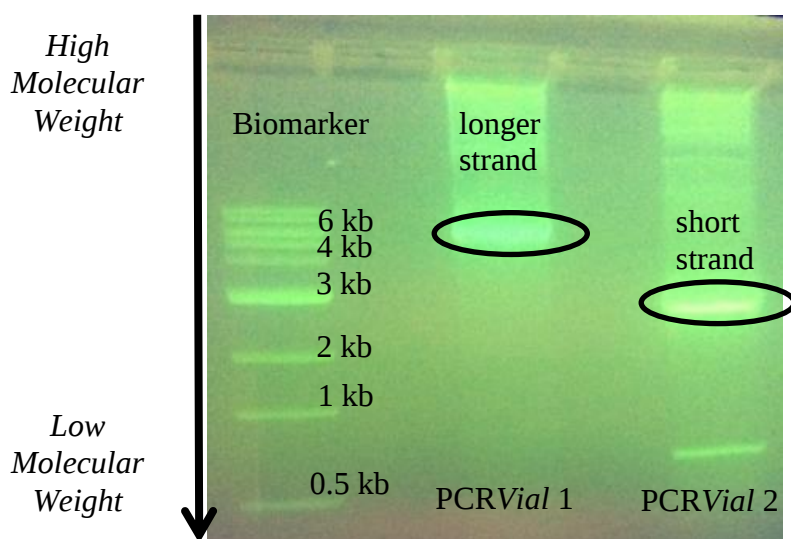


Figure 29: Bands corresponding to proper lengths of *ds*-DNA formed by initial PCR step of Gibson Assembly procedure.¹⁴⁰ The PCR of contents of two vials, labelled Vial 1 and Vial 2, results in the formation of two linear double-stranded (*ds*) DNA. Each vial contains a set of appropriate mutation and corresponding Gibson primers. Vial 1 contained P508A Forward primer with *pMAK* Rev primer to yield 5896kb *ds*-DNA segment and vial 2 contained P508A Reverse primer with *pMAK* Fwd primer to yield 2568kb *ds*-DNA segment as theorized in Figure 25. Primer sequences are given in Table 11 while PCR conditions are given in Table 10. The bands were collected by gel extraction and subsequently submitted for sequencing.

After a full genetic sequencing read confirmed the intended P508A mutation was in place in the FTH004 cells, the variant *E. coli* was grown in Luria Broth with appropriately added nutrients (Appendix 7).⁵⁷ A batch of the FTH004*E. coli* was grown similarly. Optical density measurements at 600 nm (OD_{600}) were taken hourly until growth reached the stationary phase where expression of Hyd-1 is maximum.¹⁴³ The growth curves were indistinguishable from each other as shown in Figure 30. Thus, the mutation did not unintentionally interfere with the normal growth of *E. coli*.

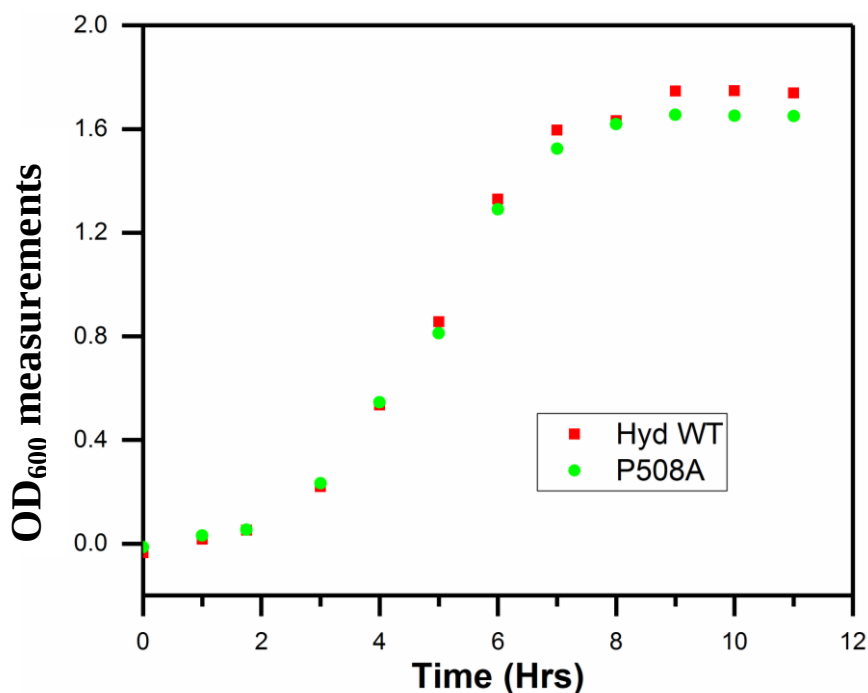


Figure 30: Growth curves of *E. coli* of WT/native and mutated P508A variant. Optical measurements at 600nm were carried out hourly until the stationary phase in growth was reached where expression of Hyd-1 is maximum.¹⁴³ The introduction of the mutation did not interfere with the normal growth as observed by the similarity in the profiles of the WT compared to that of the mutated variant. The *E. coli* were then collected for further processing to isolate and purify the enzyme.

3.2.3 Purification of Variant Enzyme

A more detailed description of the growth of *E. coli* and subsequent isolation and purification of the enzyme is given by Lukey *et al.*⁵⁷ It is reproduced briefly here while the full protocol is given in Section 8.5.4.

Once the stationary phase of *E. coli* is obtained from growth under anaerobic conditions, the *E. coli* culture is centrifuged to collect the cells followed by addition of lysozyme and DNAase to resuspend the cells. These steps and the ones following were performed (aerobically) at 4°C. After passing through a French Press, the membrane fraction was collected by centrifugation and homogenized. Subsequent solubilizing of

the membranes with 30% Triton X-100 detergent resulted in a suspension of the membrane fraction which can be passed through an Äktapurifier system, a UV system, whereby the product can be monitored by following two wavelengths: 280 nm, the wavelength at which the peptide and aromatic bonds within the protein scaffold absorb, and 420 nm, where the Iron (Fe) absorb. The Äktapurifier system was fitted with a nickel (Ni)-high affinity column. A Ni-column is appropriate for immobilizing the enzyme from the suspension as a hexa-histidine tag is encoded by the chromosomal sequence and ultimately expressed in the product. The enzyme was then eluted into fifty 1.5ml fractions by elution with imidazole gradient. Inspection of the Äktapurifier trace suggests that fractions 10–30 correspond to proper formation of protein with Fe incorporation. Six (6) fractions within the fractions 10–30 (Figure 31) were collected. These 6 fractions were subjected to denaturing electrophoresis (Section 3.2.4) for checking the proper formation of the HyaA and HyaB subunits of the enzyme. After confirmation that all six fractions had properly formed HyaA and HyaB subunits (suggested by comparison of migration to WT Hyd-1 from *E. coli*; Figure 32), the entire range of 10-30 fractions were pooled for characterization by PFE. Previous samples of WT Hyd-1 subjected to PFE have been known to incorporate HyaA, HyaB and HyaC units though experience within the groups indicate that the presence/absence of HyaC does not alter the electrocatalytic profiles obtained during PFE measurements. The presence of HyaA and HyaB is imperative to getting proper profiles.^{19, 34a, 57} While preparations for PFE (as per standard lab protocol for PFE experiments (Appendix 7)) contain combinations of HyaAB and HyaABC, those for forming crystals (Appendix 7) are subjected to extra steps of size exclusion and hydroxyapatite purification and thus, HyaC is not present in them. The purification needed for crystallization was carried out by Dr Stephen Carr (Research Complex at Harwell (RCaH), Didcot, UK).

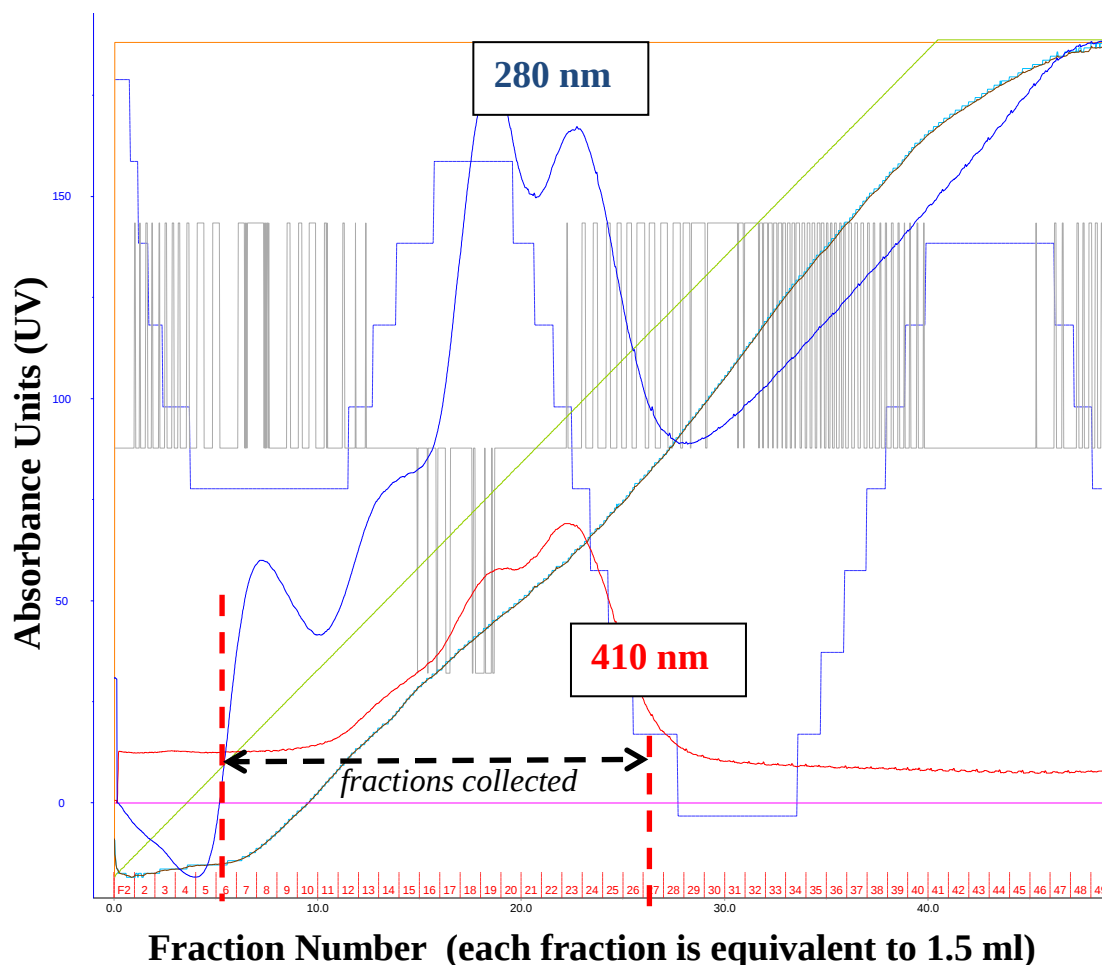


Figure 31: The profile of elution from Äktapurifier. The Äktapurifier system was fitted with a nickel (Ni)- affinity column which immobilizes the enzyme (containing a hexa-histidine tag to enable such immobilization). The enzyme is then eluted into fifty 1.5ml fractions while the purifier system uses UV to follow two wavelengths: 280 nm and 420 nm, an overlap indicating proper protein formation with (Fe) incorporation. Based on this profile, 6 fractions within the 10-30 range were then subjected to denaturing electrophoresis to confirm the presence of hyaA and hyaB subunits. After confirmation, the range of fractions with proper formation of the hyaA and hyaB subunits was collected and pooled to characterize by PFE.

3.2.4 Denaturing electrophoresis (SDS-Page)

The six fractions collected as mentioned in Section 3.2.3 were run on a sodium dodecyl sulfate (SDS) gel: three of them are shown in Figure 32. It was found that the gel results from P508A are indistinguishable from WT Hyd-1 during this denaturing electrophoresis. The large subunit (HyaB) and his-tagged small subunit (HyaA^{His}) of

P508A formed bands identical to that of WT (HyaB: 60kDa and HyaA^{His} : 32 kDa)¹³⁶ indicating that both were processed similarly. The similarity in the gel profiles provided guidance to which of the fractions eluted from the Äktapurifier to collect (in this case, the entire 10-30 range) and confidence in the proper structural formation of the pooled enzyme. The absence of the HyaC in the figure is noted for both gels and may be attributed to the conditioning size exclusion column and hydroxyapatite columns steps which are carried out during preparing samples for crystallization (carried out by Dr. Stephen Carr).

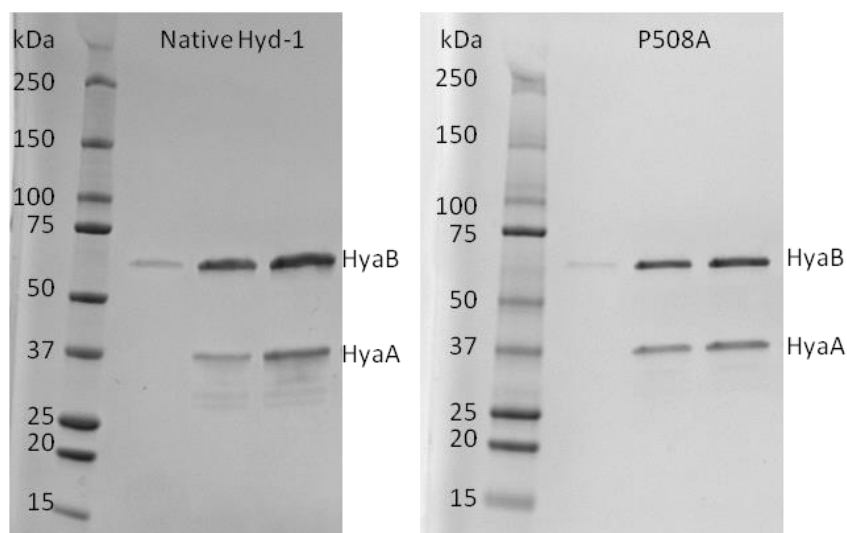


Figure 32: Denaturing (SDS) polyacrylamide gel electrophoresis of WT Hyd-1 and P508A. Each enzyme was loaded following elution from conditioning size exclusion column and hydroxyapatite columns which follow isolation and purification from the Ni- affinity column (Äktapurifier). Protein bands corresponding to the mass expected for the large subunit, HyaB, at 60 kDa and small subunit, HyaA, at 32 kDa¹³⁶ are indicated for both WT Hyd-1 and P508A; they are identical to one another.

3.2.5 Solution Assay Results

Ten µl of the pooled sample were measured for protein concentration by Bradford Assay to allow for measurement of activity of the enzyme samples per milligram.¹⁵⁰

Initial-rate activities at pH 6.0 for both the P508A variant enzyme and WT Hyd-1 were then measured by conventional steady-state assays in (initially) H₂-saturated solution whose theory and protocol are detailed in Section 2.2.5 and Section 8.5.5, respectively. The results are displayed in Table 2. The values were also converted to turnover frequencies k_{cat} (per enzyme molecule) as experiments with P508A mutant and WT Hyd-1 were carried out under 100% H₂ headspace gasflow. This should have provided saturating conditions assuming V_{max} is normally achieved in concentrations greater than ' $5 \times K_{\text{M}}^{\text{H}_2}$ ', whose value was derived in Section 3.3.

Table 2: Activity and turnover frequency of P508A canopy variant. The results were obtained by absorbance measurements of methylene blue at 600 nm and compared that of WT Hyd-1 from *E. coli*. Both samples were at pH 6. Errors given in parentheses are the standard error of the mean of at least three repeats. The values associated with the residue P508A work is a product of work carried out for this thesis (in conjunction with Dr. Rhiannon Evans) while those WT Hyd-1 are quoted from paper by Brooks *et al.*^{126a}

PARAMETER	WT HYD-1	P508A
Solution assay, $\mu\text{mol H}_2 \text{ min}^{-1} \text{ mg}^{-1}$ enzyme	153.9 (± 18.2)	70.3 (± 5.8)
Solution assay turnover frequency (s^{-1})*	257.4 (± 30.4)	117.6 (± 9.7)

* under 100% H₂, the turnover frequency corresponds to k_{cat}

3.2.6 Crystallization Data

The P508A variant of Hyd-1 was purified aerobically and crystallized as described previously.^{126b} Crystallization were performed by Dr. Stephen Carr– his report on crystal formation and modelling are briefly reproduced here along with the Figure 33 which was made by him.^{126a} Long, rod-shaped crystals of P508A grew within 24 h and diffracted X-rays to a maximum resolution of 1.2 Å. It was confirmed that the P508A

mutation was indeed successful and the protein architecture was unchanged relative to WT Hyd-1 and the polypeptide backbones could be superposed with an r.m.s.d. of 0.47 Å (Figure 33). Although the overall structure was unchanged, some local changes were observed and are detailed below.

Several side chains immediately adjacent to the active site displayed conformational heterogeneity, including residues C76 and C576 (thought to hold the nickel in position in WT) which were observed in two varied conformations: occupancy refinement showed the ‘expected’ conformation (pointing to the metal) of these residues at 80% of the time while at remaining 20% of the time, the residues point away. Interestingly, the occupancy of the Ni ion was also found to be approximately 80%. This should partly explain why the turnover frequency of P508A, as measured by solution assays, is ~54% lower than WT Hyd-1 from *E. coli*.

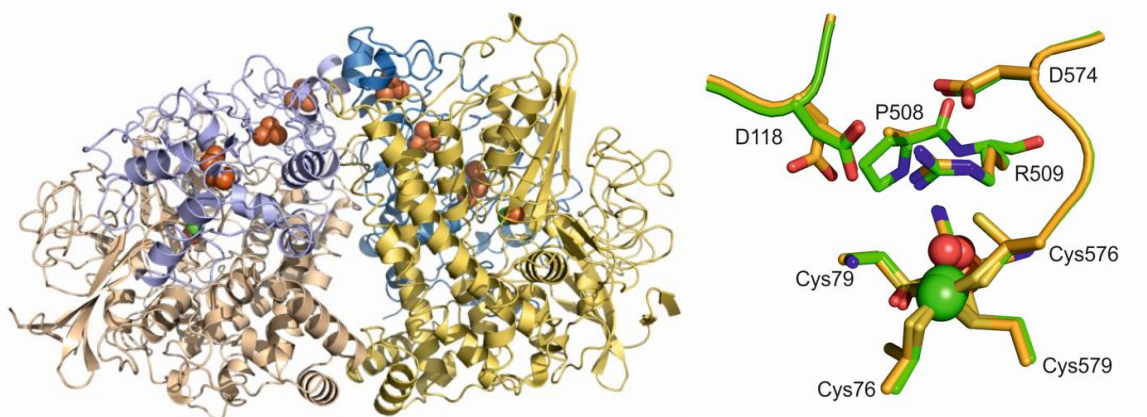


Figure 33: Overall Structure of P508A (PDB code: 5JRD). (A) The comparison of overall structures of Hyd-1 P508A shows no significant changes to the protein structure when compared to WT enzyme. The small subunit chains are coloured blue and purple, and the large subunits gold and fawn. The Fe-S clusters and [Ni-Fe] centre are shown as spheres. This figure is reprinted (with permission) from a study on Hyd-1 and some of its mutant variants by Brooke *et al.*^{126a} (B) A close-up of the active site shows that neither the alanine residue (in yellow) nor the proline residue (blue and green) in 508 position displace the arginine residue (509 position) with respect to the bimetallic site. The picture was kindly provided by Dr Stephen Carr.

The D118 side chain was also observed in two conformations: the first formed a salt-bridge with R509 and the second was rotated by approximately 60° to form a hydrogen bond with a water molecule that fills the cavity created by the P508A mutation and the main chain carbonyl group of V78. The D118 residue was thought to be important in stabilization of the arginine residue but was discounted⁴⁴ in favour of a hypothesis which supports its proton-transferring role (unpublished work of FAA Group). The introduction of heterogeneity in D118 residue conformations upon carrying out P508A mutation may account partly for the loss in its activity compared to that of the WT (as measured by solution assays). However, the distance of the arginine residue to the bimetallic centre is the same as in the WT, discounting the role of proline in pre-organization of the arginine residue.

Table 3 shows the average temperature factor values of the P508A variant (2nd column) in comparison with those obtained with other canopy mutants prepped similarly (3rd – 5th column). It can be seen that in the crystal structure of P508A, the average temperature factor for each residue in the canopy region of the active site is higher than in the other variant structures of comparable resolution. An elevated temperature factor suggests the flexibility of the residues are higher –this fact and the conformational heterogeneity of several residues and an elevated average temperature factor of the other residues in the canopy region indicates that the proline plays an important part in maintaining local stability.

Table 3: Average temperature factors for residues at the catalytic centre of P508A variant. All values calculated using B-average from the CCP4 suite of programs. The values associated with the residue P508A work is a product of work carried out for this thesis while those of the other residues are quoted from paper by Brooks *et al.*^{126a}

RESIDUE	P508A	D574N	D118A	D118N/D574N
Resolution/Å	1.2	1.23	1.25	1.1
118	16.3	10.8	8.9	7.7
508	11.7	8.4	8.7	7.6
509	13.5	9.0	14.0	9.6
574	14.3	9.8	11.1	8.2

3.3 Results of PFE experiments

The results of protein film electrochemistry (PFE) experiments are given here- it is possible to calculate a number of reactivity characteristics and comparisons were carried out between the results obtained with P508A variant enzyme and the WT to yield information about the role of proline in maintaining the pre-organization of the arginine residue at the 509 position. The theories and protocol behind application of PFE to measuring these parameters are covered in Chapter 2 (Section 2.1.4). Initially, a slow CV is run at 5 mV s^{-1} from -0.68 V to $+0.24 \text{ V}$ as shown in Figure 34: this slow scan reveals that the overpotential requirement for the WT and the P508A variant is the same in the direction of oxidation of hydrogen.

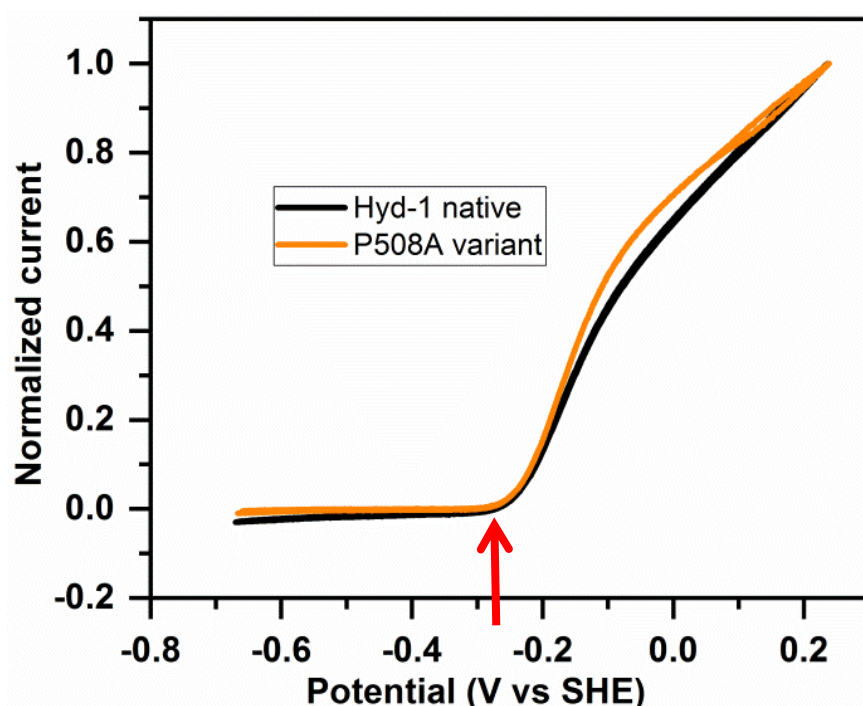


Figure 34: Slow scans of P508A compared to that of WT Hyd-1. Cyclic voltammograms were scanned between -0.68 V and $+0.24$ V at a scan rate of 5 mV s^{-1} whilst maintaining 100% H_2 in the headspace. Both enzymes show the same onset potential for hydrogen oxidation (red arrow). Experimental conditions: pH=6, $T=30^\circ\text{C}$ and rotation rate=3000rpm.

Overpotential is crucial in indicating changes to mechanism occurring at the active site and the results demonstrate that the replacement of the proline residue with a smaller alanine residue does not change the mechanism occurring— provided arginine is responsible for activation as proposed by Evans *et al*⁴⁴, the smaller alanine residue (flexible) does not seem to increase/decrease the driving force requirement when compared to the larger proline residue (rigid structure).

Measurement of $K_M^{\text{H}_2}$: Before each experiment, activation of film was carried out. Activation was needed to confer as much activity as possible to the enzyme sample as some of it had inevitably converted to an oxidized inactive state during the isolation and purification stages (Section 8.1). Cyclic voltammograms were scanned between -0.65

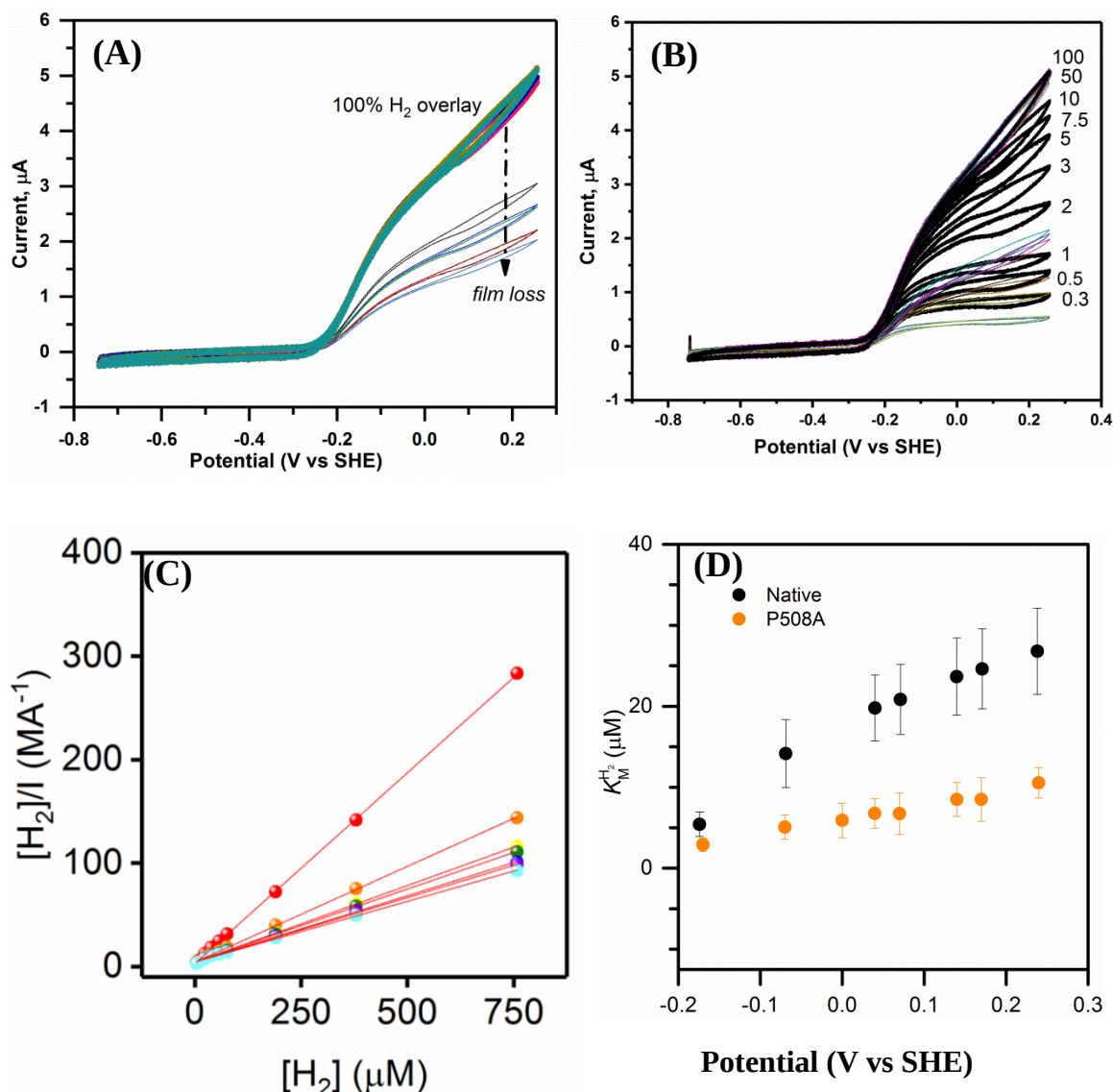


Figure 35: (A) Overlay of scans carried out with 100% H_2 in headspace gasflow. Film loss ‘corrections’ were carried out which caused the scans to overlay demonstrating the appropriateness of the corrections applied. The same corrections can then be applied to scans carried out at other partial pressures of H_2 in the headspace to give the value of ‘ i ’ to be used for calculating $K_M^{\text{H}_2}$. (B) Variation of current responses with variation in concentrations of H_2 in the headspace gasflow, with film loss correction (thick black lines) and without film loss correction (faint coloured lines). (C) The value of ‘ i ’ from the various partial pressures of H_2 in the headspace can be used to calculate $K_M^{\text{H}_2}$ by Hanes-Woolf analysis (detailed in Appendix 4). (D) Potential dependence of $K_M^{\text{H}_2}$ of WT Hyd-1 and P508A variant. The values associated with P508A is product of work carried out for this thesis while those of the WT are quoted from paper by Brooks *et al.*^{126a} Error bars represent the standard error of the mean of at least three repeats. Experimental conditions: pH 6, T= 30°C, rotation rate = 3000 rpm.

and +0.24 V at a scan rate of 5 mV s⁻¹ whilst varying the H₂ concentration in the range 0.3 to 100%, with Ar used as a carrier gas. Film loss calculations carried out as per Appendix 3 protocol for scans carried out at 100% H₂ flows in the headspace show an agreement of the overlays (Figure 35A) ensured confidence that the correction formula was correct. The correction was then be applied in the time frame of the experiment to other partial pressures of H₂ in the headspace.

The correction applied for other concentrations are shown in Figure 35B: light colours indicate original scans while the darker colours indicate the corrected versions. The $K_M^{H_2}$ was subsequently determined from these scans by Hanes-Woolf analysis (Appendix 4) for data in the range -0.17 to +0.24 V. Data below this range was discounted as low currents at these potentials resulted in poor signal-to-noise ratio.

A comparison of the variance of $K_M^{H_2}$ with potential between P508A and WT Hyd-1 is shown graphically in Figure 35. Notable about the results is the fact that, though the absolute values of the $K_M^{H_2}$ do not differ widely between that of the P508A variant and the WT initially, that of the variant remains constant throughout the potential range examined while that of WT increases rapidly with increase in driving potential.

Given that the onset potential is the same for the two enzymes as seen in Figure 34 (red arrow), the overpotential applied to each of them is the same at any given applied potential. The $K_M^{H_2}$ dependence on potential is generally a reflection of the dependence of k_{cat} on the applied potential: k_{cat} of the WT is potential – dependent whereas that of P508A variant is not.

Measurement of E_{switch} : The electrocatalytic E_{switch} (as discussed in Section 2.1.4) profiles of a slow reverse scan from high potential to low potential, as determined by PFE, are shown in Figure 36. The profiles were obtained by subjecting a film of the enzymes on PGE electrode to an initial high potential (+0.391 V vs SHE) and pH9 under Argon (Ar) atmosphere for 10,000 sec to fully ‘inactivate’ the enzyme⁴⁴ and then a slow scan (0.1mV/s) was carried out from the high potential to the low potential as this would ‘activate’ the enzyme to the oxidized active state, Ni_a-Si_a. The profiles of P508A were normalized to that of WT Hyd-1 to allow comparison. The insets of Figure 36B show derivatives of the current profiles to reveal the point of maximal reactivation known as the E_{switch} . A result of 3 repeats each for both WT and P508A variant gave that E_{switch} for Hyd-1 as 0.24 ± 1.69 V and that for P508A as 0.20 ± 1.32 V which indicates that the transition from a proline residue to alanine alters the ‘ease’ with which the oxidized ‘inactive state’ of the enzyme is formed. A ~40 mV stabilization effect of the oxidized ‘inactive state’ is observed.

This may be understood by the fact that neutralizing the negative charges of nearby aspartate residues also stabilize the formation of the oxidized ‘inactive state’ of the enzyme.^{126a} The alanine residue may be bringing about the same effect by increasing the flexibility of the arginine residue it holds and thus decreasing the ‘reach’ of the negative charges of the same aspartate residues.

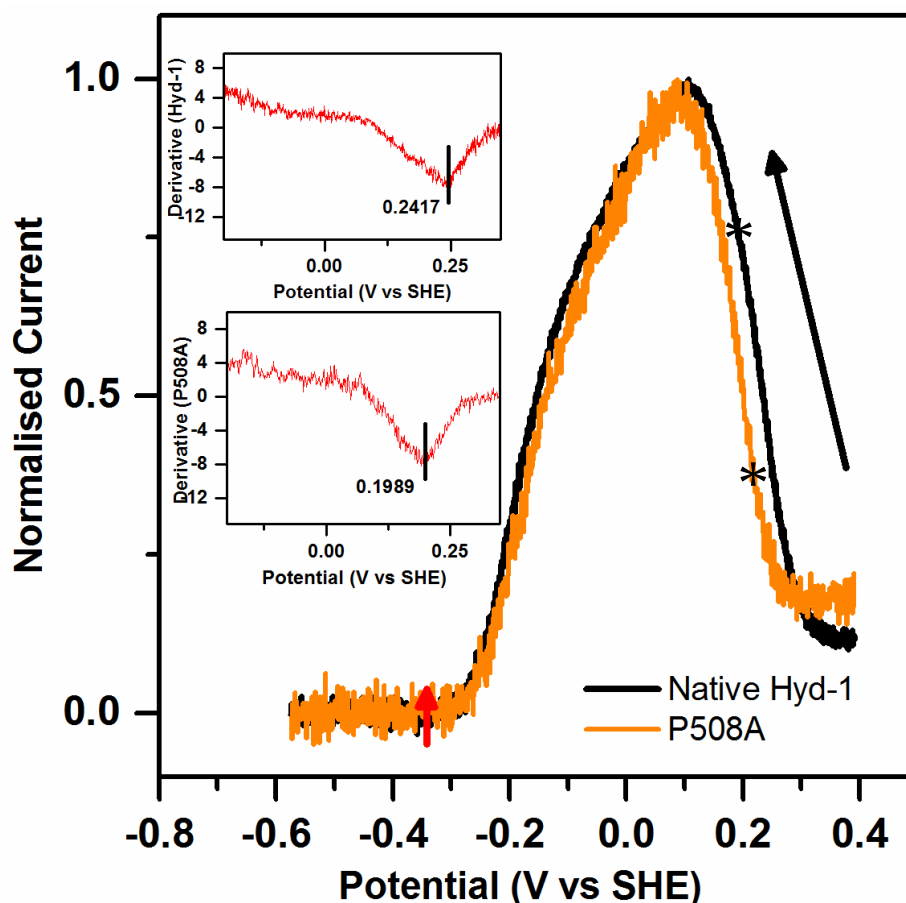


Figure 36: Electrocatalytic profile of P508A compared to that of WT Hyd-1. The enzyme films were exhaustively inactivated at high potential (+0.391 V) and pH 9 under an atmosphere of Ar for 10,000 s before the potential was scanned from the high potential to low potential at a slow scan rate (0.1 mVs^{-1}) under 100% H_2 to yield the electrocatalytic profile. The voltammograms were normalized to allow comparison. Experimental conditions: pH 6, $T = 30^\circ \text{C}$, rotation rate = 3000 rpm. The points marked with * indicate the E_{switch} values.

An alternate explanation may be in a finding by Leger *et al* that the reactivation process, while being affected by the reduction potential of the oxidized state to the reduced state, may also be affected by the electronic relay system (Fe-S clusters) that connect the active site to the electrode.¹²⁹ Since the Fe-S clusters are known to be responsible for O_2 -tolerance of the Hyd-1 enzyme, the P508A variant was subsequently examined for variance in behaviour with WT when exposed to O_2 , either in a transient or prolonged manner.

O₂ Inhibition (Transient and Prolonged Exposure): The response of the P508A variant to transient O₂ exposure was assessed, as described previously, with oxygen-saturated buffer injected at +0.03 V during a potential sweep from −0.40 V to +0.24 V at 5 mV s^{−1} to give a solution concentration of 154 μM O₂.^{19, 34a} The constant flow of H₂ flushed O₂ from the system, with complete removal upon the start of the reverse sweep to negative potentials. The current dropped within 2 s of O₂ injection (Figure 37A) and by the time reverse sweep was initiated, rapid recovery of substantial H₂ oxidation current was seen. This experiment demonstrates that the P508A variant is also O₂ tolerant as is the WT.

The effect of more prolonged exposure to O₂ on the P508A variant was assessed at 0 V under a continuous O₂ flow through the headspace in 10% H₂ with Argon being the carrier gas and making the balance. (Figure 37B). The variant enzyme was able to sustain H₂ oxidation as the O₂ concentration was progressively increased even to 10% O₂–90% H₂ composition (equivalent to 118 μM O₂ concentration) as is reported of the WT.^{19, 34a} The O₂ flow through the headspace were ceased and returned to 100% H₂ (to remove O₂) and it was seen that the current began to recover spontaneously to a significant level.

Since the ability of Hyd–1 to be O₂-tolerant arises from the furnishing of electrons by the proximal Fe–S cluster.^{19, 34a} the results of O₂-tolerance experiments is not surprising as the point of mutation is far from the location of the Fe-S cluster. Furthermore, it gives the confidence that the proline residue did not have an unintentional effect on the Fe–S cluster effect.

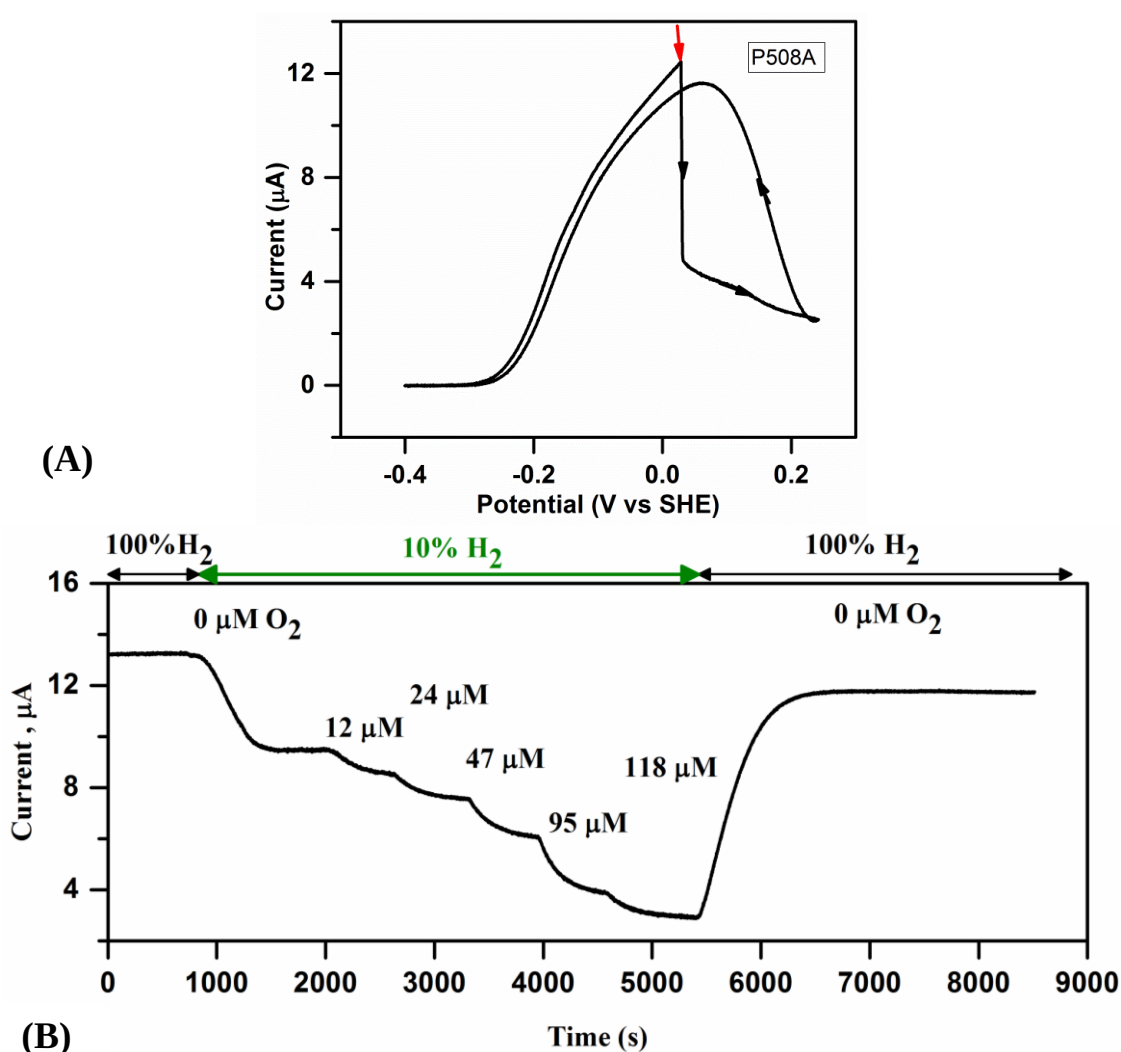


Figure 37: (A) Effect of transient exposure to oxygen on the H_2 oxidation activity of the P508A variant. Cyclic voltammograms were performed between -0.40 V and $+0.24$ V at a scan rate of 0.5 mV s^{-1} under a constant headspace gas of 100% H_2 . The O_2 -saturated buffer was injected (red arrow) at $+0.03$ V to give a total O_2 concentration of $154 \mu\text{M}$. The constant H_2 flow into the cell headspace and slow scan rate ensured all O_2 is flushed from the system by the time the reverse scan begins. (B) Effect of continuous exposure to oxygen on H_2 oxidation activity of the P508A variant. A chronoamperometry experiment measured the current at 0 V in 100% H_2 for the first 700 s before stepping to 10% H_2 . After further 1100 s, O_2 was added to the headspace and the concentration increased at successive 600 s intervals until 10% O_2 concentration in the headspace was reached [$118 \mu\text{M}$]. The O_2 flow was then stopped and the headspace gas returned to 100% H_2 to trace the recovery of activity. Experimental conditions: pH 6, $T = 30^\circ\text{C}$, rotation rate = 3000 rpm.

Sensitivity to CO: WT Hyd-1 is only slightly inhibited by CO, with a small drop in catalytic activity when CO is first introduced.⁵⁷ To compare inhibition by prolonged exposure to CO, the catalytic activity of P508A was monitored as current at -0.06 V as done previously.¹⁹ The potential was chosen to be high enough to see oxidative catalytic current but not so high that the oxidized 'inactive' state starts to form.

An initial baseline under 100% H₂ was recorded in a chronoamperometry experiment in which the system was then changed to 20% H₂ with Ar as the carrier gas such that the total gas flow was maintained (Figure 38A). CO was then introduced and then the partial pressure was increased stepwise to reach concentrations of 27, 44, 71, 88, 133 and 179 μ M in the cell solution (calculated by Henry's law) and a similar profile from P508A was obtained as is reported of WT Hyd-1.¹⁹ Turning off the flow of CO resulted in the flushing out of CO with 20% H₂ and led to the observation that the majority of the activity recovered upon its removal as occurs in the case with WT Hyd-1.¹⁹ The CO inhibition experiment was done as a means for testing that the proline to alanine substitution did not accidentally introduce blockages/widen the gas channels that lead to the active site and provide confidence in the results obtained in experiments such as the solution assay experiments.

Figure 38B compares Hyd-1 and P508A in their responses to presence of CO with that of a system known to be sensitive to CO, that is, with Hyd-2. The steepness of the plot of $\frac{i_{max}}{i}$ vs. [CO] according to Equation 22 indicates similar sensitivity to CO of the P508A variant and the WT Hyd-1. Thus, the proline substitution to a much smaller alanine residue does not change the insusceptibility to CO.

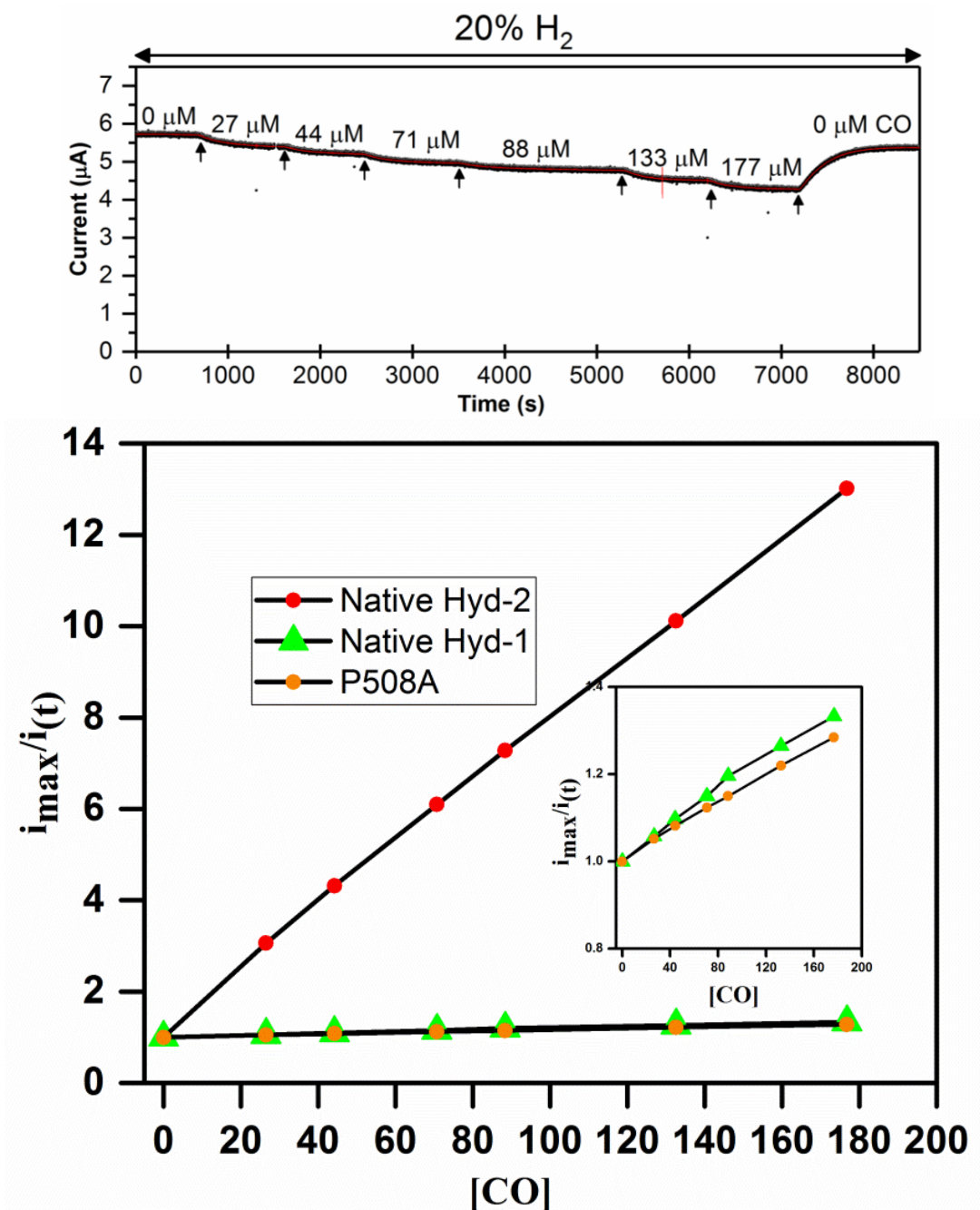


Figure 38: (A) Effect of continuous exposure to CO on the H_2 oxidation activity of P508A variant. Chronoamperometry was carried out at -0.06 V and 20% H_2 as previously described.¹⁹ After 700 s, CO was added to the headspace and the concentration increased until 20% CO was reached whilst still maintaining 20% H_2 (balance provided by Ar). Experimental conditions: pH 6, 30°C , rotation rate = 3000 rpm. (B) CO inhibition of H_2 oxidation activity of WT Hyd-2, WT Hyd-1 and P508A variant of Hyd-1. Plots of i_{max}/i vs $[\text{CO}]$ assess the sensitivity to CO inhibition of Hyd-2 (red), Hyd-1 (green) and P508A variant (orange). The P508A result is a product of the work of this thesis whereas the Hyd-1 and Hyd-2 results are reproduced from study by Brooke *et al.*^{126a}

Enthalpy: Activation enthalpies for H₂ oxidation, $\Delta H^\ddagger$, were determined from the temperature dependence of electrocatalytic currents recorded during cyclic voltammograms under 100% H₂ and shown in Figure 39 as a function of potential. WT Hyd-1 and P508A show little variation in $\Delta H^\ddagger$ over the potential range -0.1 to +0.24 V providing further proof that the proline to alanine substitution did not interfere with the mechanism undertaken at the active site. To derive this graph, Eyring plots were initially made (Appendix 8). Generally, temperature ranges of 10–45°C is used in PFE experiments, but for P508A mutant a temperature range of 10–65°C was used to test the hypothesis that the substitution of proline with alanine would result in the destabilization of the protein which will manifest at higher temperatures. A set of experiments were carried out by scanning CVs between -0.66 and +0.24 V at a scan rate of 5 mV s⁻¹ at various temperatures and regularly returning to 30°C to allow for film loss correction.^{34b} (Appendix 3) The Eyring plot obtained from use of 10–45°C range for WT Hyd-1 were similar to 10–65°C range for P508A mutant and the potential dependence of the activation enthalpies for H₂ oxidation, $\Delta H^\ddagger$, are the same for both the enzymes. This demonstrates that the local instability (higher average temperature factors and heterogeneity in conformation of several residues as seen by crystallization) does not translate to instability of the whole protein.

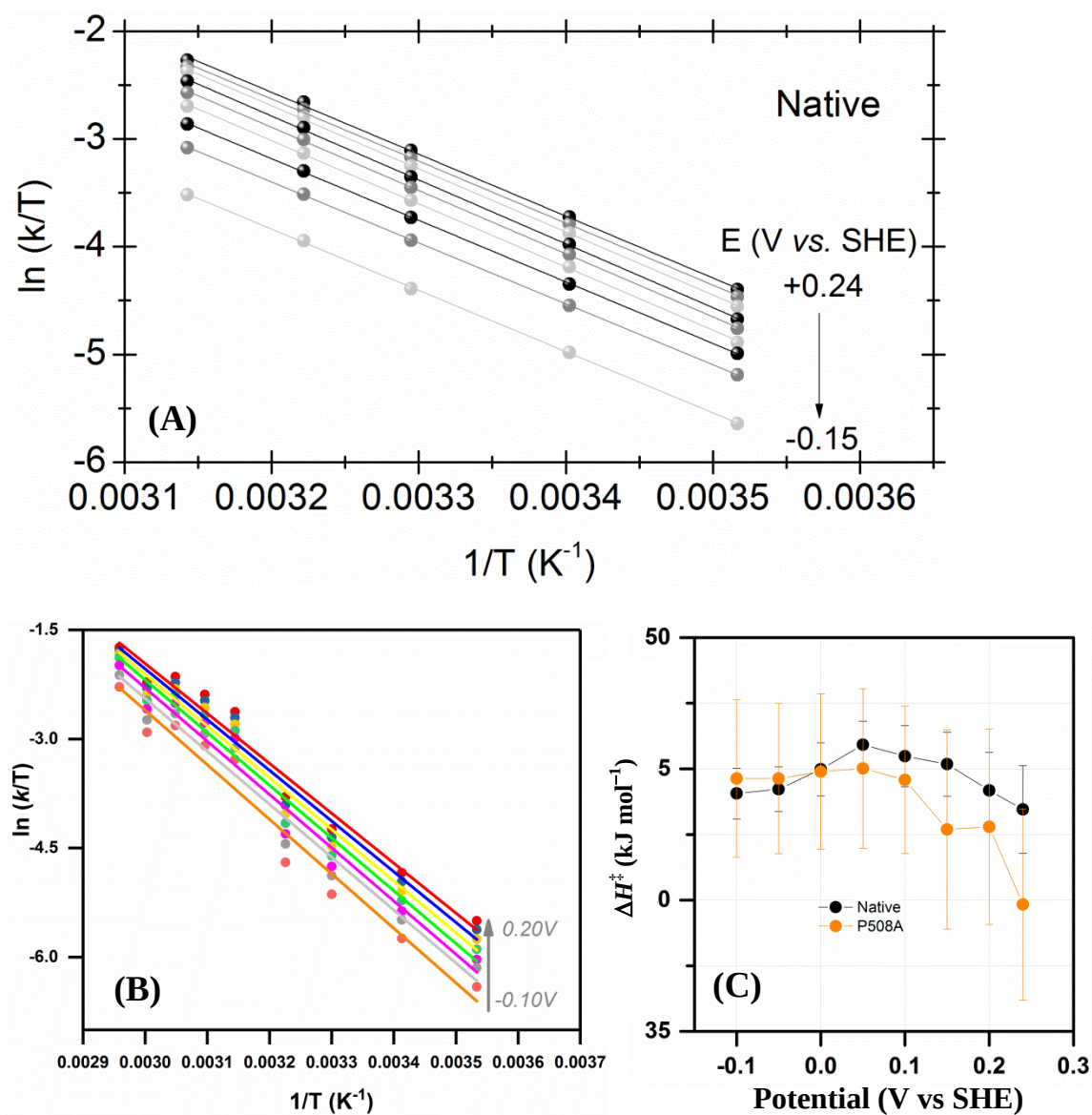


Figure 39: Potential dependence of the activation enthalpy ($\Delta H^\ddagger$) of WT Hyd-1 and P508A variant. Cyclic voltammograms were scanned between -0.65 and $+0.24$ V at a scan rate of 5 mV s^{-1} at five different temperatures in the range 10 – 45°C (internal electrochemical cell temperature). The average values for P508A variant include data collected at extended temperature range of upto 65°C . Prior to each scan, the temperature equilibration was monitored by measuring cyclic voltammograms at 30 mV s^{-1} until they overlapped. After each 5 mVs^{-1} scan, the temperature was returned to 30°C so that film loss could be accounted for (Appendix 3). Eyring plots of (A) Hyd-1 WT (kindly provided by Dr. Rhiannon Evans and Emily Brooke of FAA group) and (B) P508A mutated sample (this work) with potential values and ' i ' values from CVs give the variance of activation enthalpy with potential. (C) Potential dependence of the activation enthalpy ($\Delta H^\ddagger$) of WT Hyd-1 (black trace) and P508A variant (orange trace). Error bars represent the standard error of the mean of at least three repeats. Experimental conditions: pH 6, rotation rate = 1000 rpm , $100\%\text{ H}_2$.

H₂ production: Cyclic voltammograms in the potential range -0.56 V to $+0.26$ V were recorded at a scan rate of 10 mV s^{-1} at pH 3 and 1% H₂ (Figure 40). As all other variants reported by Evans *et al*⁴⁴, P508A retained the ability to form H₂ at low pH and low H₂ concentration which initiates at the same onset potential as WT Hyd-1. Given that the proline residue is being investigated for their role in holding arginine in place, it is essential to know that the mechanism of H₂ activation, in either the oxidation direction and the reverse H₂ reduction direction is not perturbed. While the initial slow scan shows that the onset potential in the oxidation direction is unchanged between the WT Hyd-1 and the P508A variant, this experiment demonstrates that the onset potential in the reduction reaction is also unchanged. The mutation does not interfere with the H₂ activation mechanism in any way that influences the driving force requirement.

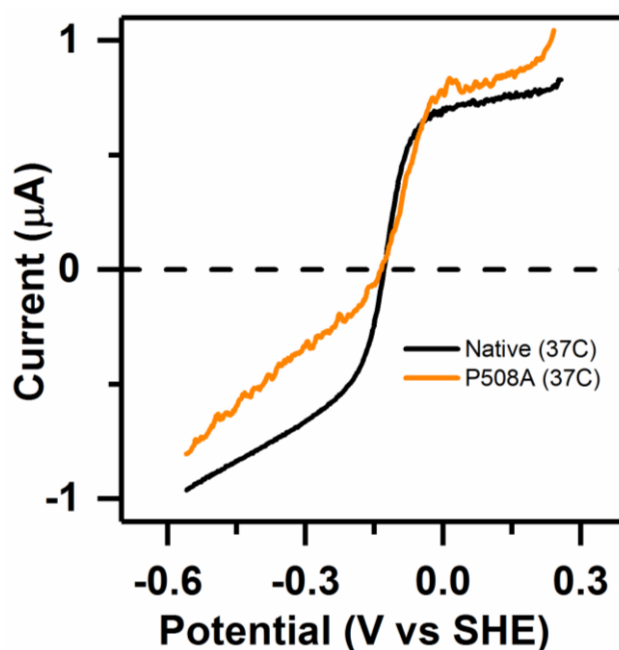


Figure 40: Hydrogen production by WT Hyd-1 and P508A variant at pH 3 and 1% H₂. The potential was cycled between -0.56 and $+0.24$ V at a scan rate of 10 mV s^{-1} . The current magnitudes are dependent on the film quality and history and should be not taken as a quantitative determination of relative ability to produce or oxidize H₂, but to note are the retainment of the ability to produce H₂ by the P508A variant and the similarity in the onset potential between the WT Hyd-1 and the P508A variant. Experimental conditions: $T = 37^\circ\text{C}$ and rotation rate = 2000 rpm.

pH optima experiments: Cyclic voltammograms of P508A were carried out at different pH and it can be seen in Figure 41A that they cut the x-axis at more negative values as pH is increased. This is as per expectation by Nernst equation.

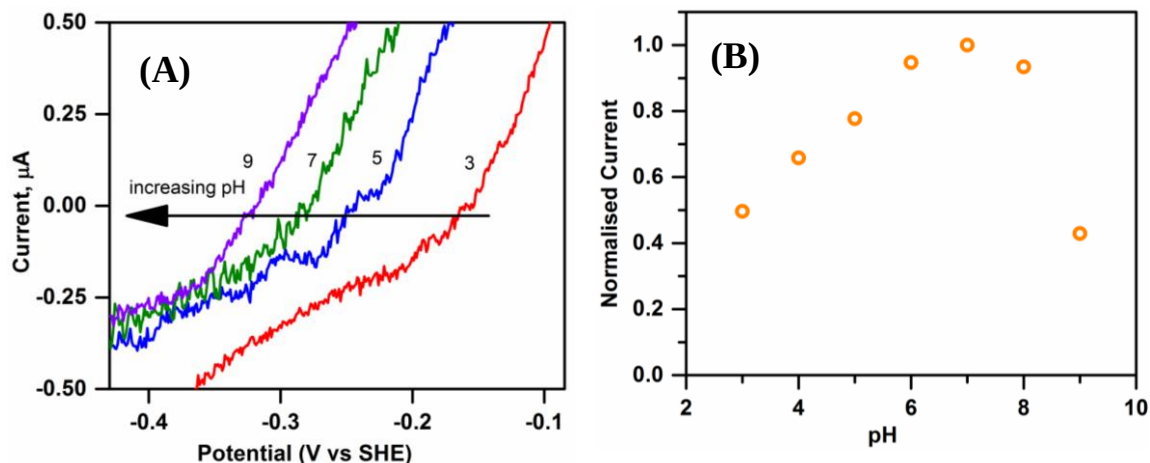


Figure 41: (A) X-axis intercept of cyclic voltammograms of P508A carried out at different pH. Initially, a CV was carried out of P508A variant enzyme coated onto PGE electrode in a cell buffer solution at pH 6.0. Then CVs were recorded at different pH (pH 3–9) of the electrolytic solutions ensured with buffer exchanges being carried out prior to recording CVs. The plots were zoomed into to display the points at which they intercept the x-axis. It is seen that the values of the intercepts occur at more negative values of potential as pH is increased in the range pH 3.0–9.0 as per expectation by Nernst equation. (B) Determination of the pH optima for WT Hyd-1 and P508A variant. Chronoamperometry experiments at 0 V vs SHE were performed in 100% H_2 . Initially the cell buffer solution was pH 6.0 and once the current had stabilized in chronoamperometry and recorded, it was exchanged for different pH buffers in the range pH 3.0–9.0. The buffer was returned to pH 6 periodically to allow for film loss correction. The current at each pH was then normalized to the highest current in the different pH range. Experimental conditions: $T = 30^\circ\text{C}$, rotation rate = 1000 rpm, scan rate = 20 mV/s, 100% H_2 .

For integrity of all experiments, especially, in order to ensure that solution assay measurements were carried out close to optimal pH conditions, the pH dependence of activity for P508A variant was determined electrochemically from the catalytic H_2 oxidation current by chronoamperometry at 0 V vs SHE as shown in Figure 41B.

A single electrode modified with a film of enzyme was transferred from a H₂-saturated buffer solution at pH 6.0 to other H₂-saturated solutions over the pH range 3.0–9.0 while being returned periodically to pH 6.0 to allow film loss correction (Appendix 3).^{34b} The optimum pH was found to be pH = 7 similar to that reported for Hyd-1.^{126a} Thus, the rates of activities as reported by solution assay can be assumed to be a true reflection of the activity differences between P508A variant and WT Hyd-1 and not the result of enzyme samples being tested in adverse pH conditions.

3.4 Result of Differential Scanning Calorimetry (DSC)

The comparison results of a differential calorimetric scan (DSC) run on the WT Hyd-1 and P508A variant are shown in Figure 42. The Cp value (specific heat capacity value which is obtained at a constant pressure and is a measure of the heat required to raise the temperature of a sample by 1 K) is not a direct concern of this work but temperature at which peaks occur are as they are concerned with ‘unfolding’ of the protein in question. While WT Hyd-1 shows three distinct peaks, that of P508A variant shows two.

The loss of a peak may be assigned to the preparation of the samples. While WT Hyd-1 sample were formed as per standard lab protocol for PFE experiments (Appendix 7), the P508A sample had been submitted to extra steps of size exclusion and hydroxyapatite purification for crystallization preparation. These extra steps are efficient at removing the cytochrome that anchor the enzyme to the membrane *in vivo*. Thus, the peak absent from the P508A profile may be assigned to that which results from the unfolding of the cytochrome subunit.

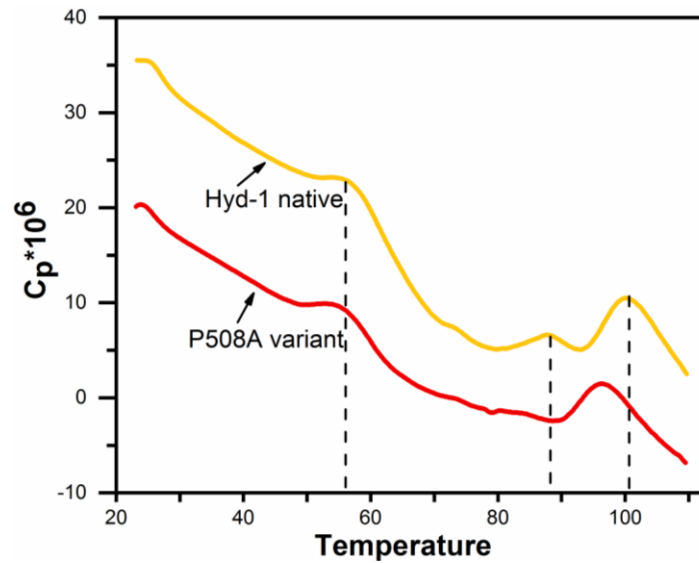


Figure 42: Differential calorimetric scan (DSC) results of WT Hyd-1 and P508A variant. The enzyme under study is placed in a sample cell and the buffer in which the enzymes are prepared (Buffer C:Appendix 9) placed in the reference cell. The temperatures of the cells are increased and when unfolding occurs, the heat absorbed by the process brings about a cooling effect of the sample cell such that extra heat needs to be supplied to it to maintain a temperature identical to that of the reference cell. The supply in heat to maintain this equilibrium is recorded and appears as peaks in the C_p vs temperature graph.

The other two peaks may be that of HyaA and HyaB unfolding– the similarity in the peak at 56°C may be assigned to HyaA unfolding since the mutation is likely to affect HyaB where it is located as opposed to the HyaA subunit . Considering this, the unfolding at 100.5 °C for WT Hyd-1 may be assigned to the unfolding of the HyaB subunit which is then seen to be moved to lower values in the P508A variant (by ~4.6°C). This DSC result supports the data from crystallization about local instability but is contradicted by the PFE temperature dependency results. However, a simple reason may be assigned to the fact that the PFE experiments were conducted between 10-65°C while DSC results demonstrate that the effects manifest at much higher temperatures.

3.5 Conclusion and Perspectives

Sequence alignment of Hyd-1 with other O₂-tolerant [Ni-Fe] hydrogenases indicates a pivotal role of a conserved proline residue in holding an arginine in position for H₂ splitting and H₂ activation. Additionally, the cyclic proline residue is at the i+1 position of type I beta turn which normally is found on surfaces instead of at deeply-buried active sites. An earlier study by Evans *et al*⁴⁴ showed that replacement of salt-bridge forming nearby aspartate residues of the arginine did not affect the placement of arginine with respect to atoms of the bimetallic centre. Given that this arginine residue has been identified as the residue to bring about H₂ activation, an investigation of the proline with site-directed mutagenesis and protein film electrochemistry was considered necessary. The proline residue was replaced with alanine, a smaller but similarly neutral molecule, which would theoretically ‘introduce disorder’ into the active site. If proven to be significant, designing biomimetic catalysts based on Hyd-1 would necessitate incorporating an outer coordination shell with an arginine-holding group to be a proline-mimic for catalysis to occur efficiently.

The results of molecular biology demonstrate that the growth of *E. coli* was not affected by the P508A mutation carried out on the HyaB sequence. The mutated enzyme could then be analyzed for thermodynamics and kinetic parameters by PFE and compared with that of the WT. First and foremost, it is necessary to evaluate the results of the pH maxima experiments of P508A variant compared to that of the WT. Similar shape with maximum at pH 7 for both enzymes and a similar loss in activity when the enzymes are transferred from pH 7 to pH 6 buffer solution provide confidence in analysis of the solution assay results and parameters being measured by PFE.

Then, the Michaelis constant, $K_M^{H_2}$, was measured as previously described.⁴⁴ The value for P508 A was lower at $5.9 \pm 1.23 \mu\text{M}$ compared to $19.8 \pm 2.13 \mu\text{M}$ for the WT. Inherently, this indicates that hydrogen binding (k_{on}) increases relative to k_{off} being favoured in the mutated sample compared to that of the WT. An increase in flexibility at the active site cannot be thought to bring about such an effect but it might be relevant that R509K showed a similar drop in $K_M^{H_2}$ values.⁴⁴ Furthermore, this variable shows a potential dependence in Hyd-1 while the dependence is not visible in the mutated version. Usually the dependence is brought about by the variance of the k_{cat} on potential, the lack of which indicates that the electron transfer step in the mechanism of H_2 activation loses significance in the P508A variant. It may be that the chemical step in H_2 activation in the P508A variant becomes rate-determining: the possibility of affecting the binding of H_2 to the N in the arginine at the 59 position cannot be discounted and would indicate a profound role of the proline holding the arginine in place. A flexible arginine may be a hindrance to the chemical step involved.

Although proline's replacement with alanine residue is unlikely to bring about the effect of favouring hydrogen binding, it may be favouring proton transfer by introduction of flexibility of the arginine residue which in turn facilitate its 'flipping' to transfer the H^+ (from the splitting of H_2) to the next residue in the proton transfer network. Alternately, the extra water molecule allowed into the structure following replacement of proline to alanine could be favouring the proton transfer. The importance of the water molecules cannot be discounted given that all crystallographic structures of hydrogenases show that the active site is highly hydrated. The position of the water molecules is preserved between the active sites of hydrogenases from various organisms including *D. vulgaris*

Miyazaki *F* (PDB code: 4U9H), *Ralstonia eutropha* (4IUD) and *Hydrogenovibrio marinus* (PDB code: 3AYZ).

The onset potential for catalytic H₂ oxidation (signifying the most negative potential at which H₂ oxidation begins) is the same between both samples: this observation is not surprising given that this property is dependent on the potential of the Fe–S clusters of the electron relay. The Fe–S cluster is located >30 Å from the place of mutation and is unlikely to be affected by the mutation. The ability to produce H₂ at low pH and hydrogen concentration is also unaffected and is also not surprising given the unlikelihood of the proline to alanine mutation affecting the potential of the entry/exit Fe–S cluster. The O₂ experiments also indicate that the Fe–S cluster is unaffected by the mutation (discussed further below).

There is ~ 40mV change in the value of the E_{switch} parameter which highlights that the arginine needs to be in position to disallow the stabilization of oxidized inactive state. This may have been brought about by increasing the flexibility of the arginine which hampers the process of salt-bridging with nearby aspartate residues. This lack of salt-bridging may have brought about a lowering of the E_{switch} values as observed in other mutation experiments when the formal negative charge of the canopy residues was decreased.⁴⁴

The similarity in tolerance to O₂ by both WT Hyd–1 and P508A was substantiated by the O₂ flow through the headspace ceasing during prolonged exposure experiment and recovery of activity of the enzyme with 100% H₂ (to remove O₂) and during the transient exposure experiment before the reverse scan began (as it may be assumed that

the O₂ had been flushed away). Furthermore, the P508A variant retains the unusual characteristic that CO is a poor inhibitor of H₂ oxidation. This is a property shared with other O₂-tolerant and membrane-bound [Ni-Fe] hydrogenases. Thus, it may be assumed that change in value behaviour of k_{cat} with potential is not brought about by changes in the Fe-S cluster or changes to the gas channel but may have been brought about by probable decrease in the formal negative charge of the active site or proton transfer becoming more favourable relative to H₂ binding (thus, facilitating proton-coupled electron transfer) and hindrance to the chemical step involved in H₂ activation.

Additionally, of interest is the nearly 50% reduction in catalytic activity [70.3 (±5.8) units for P508A vs 153.9 (±18.2) units of Hyd-1] as measured by solution assay at approximately 0 V vs SHE, particularly with lower $K_M^{H_2}$ in case of the variant. It has already been concluded to be *not* due to adverse response to the pH of the buffer nor is it the result of the difference in the driving force available to it. A change in the mechanism at the active site is also unlikely (as the potential of the reaction at the active site is unaffected). One of the significant results gleaned from crystallization results conclude that although the small and large subunits of the hydrogenase have been formed correctly the local disturbance at the active site is significant. 20% of the Ni has not been incorporated into the active site which indicates the maturation process is indeed affected by the proline to alanine substitution. Of the enzymes units which had formed correctly, considerable heterogeneity and elevated temperature factors are seen in the outer shell residues which is not present in WT Hyd-1. Some local instability has thus been introduced by proline to alanine substitution which adversely affect the performance.

The differential calorimetry scans reveal that the proline to alanine residue brings about a destabilization of 4.6°C of the HyaB unit at high temperatures of ~100°C –a destabilization value which has been previously reported upon a similar mutation in onconase¹⁵¹. However, the stability is retained upto 65°C by PFE: the non- detrimental effect of mutation on stability of P508 upto a reasonably high temperature probably explains the variation of the identity of the residue in this position across the different subgroups (Appendix 10). The effects of P508 seem mainly to arise from steric hindrance provided by its bulky pyrrolidine ring, an effect which could be fulfilled by other polar uncharged or hydrophobic residues thus; for example, valine residue 500 in the actinobacterial-type [Ni–Fe] hydrogenase from *Ralstonia eutropha* perform the role of the proline¹⁵² (Appendix 10). This provides pertinent information to obtaining the ‘*design principles*’ of Hyd-1 inspired biomimetics.

Chapter 4: PFE Studies of PceA Reductive Dehalogenase

4.0 Abstract

Chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE) are among the principal contaminants of groundwater due to their utilization in various industries. Highly O_2^- sensitive PceA dehalogenase from *Sulfurospirillum multivorans* has proven to be particularly effective in converting PCE and TCE to the less harmful form, *cis*-dichloroethylene (*cis*-DCE). This study is the first of its kind in reporting interaction of the enzyme without the use of mediators on electrodes in an electrochemical set-up – a new means of research into this relevant enzyme is thus opened. It was found that the interaction of the corrinoid active site is the same for PCE and TCE, but other substrates dichlorophenol (DCP) and dibromoethylene (DBE) caused a stabilization of the more oxidized form of the active site. The inhibition effects on this PceA of potassium cyanide (KCN) and carbon monoxide (CO), its isoelectronic form, were also investigated by protein film electrochemistry (PFE). It was found that with KCN, the inhibition is potential dependent and that cobalt in the +3 state may be reached in PFE experiments. Furthermore, this study is the first to report CO as an inhibitor of PceA dehalogenase: it was previously examined but found to have no inhibitory effects on the enzyme. A role of $(NH_4)_2SO_4$ in enabling inhibition by CO was discovered.

4.1 Introduction

Dissolved chlorinated solvents, such as trichloroethene (TCE) and tetrachloroethene (PCE), are among the most frequently detected organic contaminants in groundwater worldwide and thus pose a significant threat to drinking water supply systems.⁷⁹ The colourless PCE liquid is widely known for its use in dry cleaning of fabrics (previously accomplished by TCE). The sweet-smelling and non-flammable TCE is now used as industrial solvent. Reductive dehalogenation by PceA dehalogenase from *Sulfurospirillum multivorans* (an enzyme highly susceptible to attack by O₂) was discovered in 1994⁸⁴ and since then has been studied extensively for possible exploitation in bioremediation of PCE and TCE (the natural substrates of this enzyme) to result in the formation of *cis*-dichloroethylene (*cis*- DCE). This product is less toxic than PCE and TCE though still a concern.¹⁵³ Protein film electrochemistry (PFE) has proved to be very useful in the study of O₂-sensitive enzymes and may also prove useful in the study of PceA dehalogenase. Whereas the enzyme been studied with methyl viologen mediator while spotted onto carbon electrode, direct exchange with the electrode is yet to be observed. The inability to observe the direct exchange was attributed to the enzyme's large size.¹⁵⁴ Since PFE with Pyrolytic Graphite Edge (PGE) electrodes has successfully been utilized to study enzymes which are considerably larger in size (*e.g.*, 91.3 kDa dimeric Hydrogenase-1 from *E. coli* (Hyd-1) and 110kDa dimeric CODH from *Carboxydotherrmus hydrogenoformans* (CODH_{Ch})), it was decided to characterize PceA dehalogenase from *Sulfurospirillum multivorans* (50.3 kDa) with this method. This chapter is the first to report the enzyme's involvement in direct electron transfer with an electrode- a new and novel means of investigating the O₂-sensitive enzyme.

The potential of the enzyme in technological applications requires a careful study of inhibition processes that might be pertinent. Protein film electrochemistry allows for the studies of such processes under potential control which serves not only to provide information about the type of substances which may be inhibitory but also provides information about the oxidation states of the enzyme in the catalytic mechanism that are susceptible to such inhibitors. KCN is a reported inhibitor of PceA dehalogenase¹⁵⁵ and since it has been utilized in inhibition studies of other enzymes by PFE (*E. coli* Hyd-1 and CODH_{Ch})^{103c, 156}, it was decided to utilize KCN as the inhibitor of choice in studying PceA dehalogenase. Inhibition by CO (isoelectronic to KCN) of PceA was also observed with this method although it had been studied previously but reported as *not* having any inhibitory effects.¹⁵⁵

4.2 Results and Discussions

4.2.1 Cyclic voltammetry on PGE

To observe the direct electron exchange with an electrode, it is considered essential that the enzyme under study is adsorbed onto an electrode and none are present in the electrolytic solution. As such, the surface of the PGE electrode was abraded with sandpaper and then 1-2 μ l of PceA dehalogenase (21 μ M) was spotted onto the PGE electrode with a pipette for $\sim$ 30 s before being allowed to dry completely (a process which usually takes 1 min). Upon injection of an aliquot of TCE into the electrolytic solution (to yield a final concentration of 0.8 mM) in a three-electrode electrochemical set-up with a rotating electrode, a sharp reduction wave occurred with an onset potential of -0.380 V vs SHE at pH 7 (Figure 43). This confirms that the enzyme is able to receive electron directly from the electrode for the reduction reaction which should allow further probing into its characteristics by PFE. The figure shows cyclic

voltammograms with both stationary and rotating electrodes poised at potentials between -1 to 0 V vs Standard Calomel Electrode (SCE) but converted to the Standard Hydrogen Electrode (SHE) scale to suit reporting in most electrochemical literature.

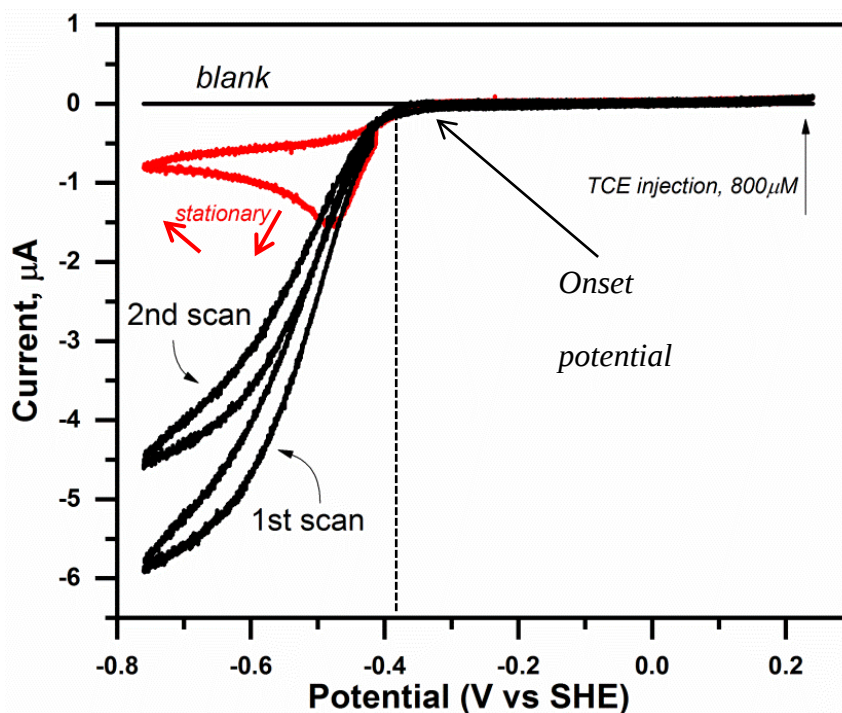


Figure 43: The reduction waves obtained with PceA dehalogenase catalyzed TCE reduction reaction. The potential was swept from positive values to negative values and then back to yield a CV. The red trace was obtained with a stationary electrode with adsorbed PceA dehalogenase and 0.8mM TCE (dissolved in ethanol) injected into the electrolytic solution. It is seen that there is an onset potential at -0.38 V and at potentials more negative than -0.5 V , mass transfer of the substrate becomes a limiting factor such the magnitude of the reduction wave decreases despite increased driving force via increased applied potential. Upon use of a rotating electrode (1000rpm), a strong reduction current was observed with the same onset potential (black trace). Experimental conditions: Buffer= 100mM TRIS+ 4mM $(\text{NH}_4)_2\text{SO}_4$, pH=7, $T = 25^\circ\text{C}$ and scan rate= 20mVs^{-1} .

The red trace was obtained with PceA adsorbed on a stationary pyrolytic graphite edge (PGE) electrode and the black trace was obtained upon rotation of the electrode at 1000 rpm. The CV obtained using a stationary electrode (red trace) increases as the potential

is swept to more negative values than the onset potential (-0.380 V) but decreases once the mass transfer of substrate becomes rate-limiting. This occurs as the potential is made more negative than -0.5 V.

A rotating electrode results in a different profile of a catalytic reduction profile becoming evident (black traces). The differences in the magnitudes of the 1st and 2nd scan obtained with the rotating electrode may be attributed to loss of substrate to the headspace, as TCE is a very volatile compound and to a much lesser extent, to film loss (Appendix 3). In previous studies,³⁰ increased surface area due to the application of nanotubes followed by covalent linking of the enzyme to this surface was an approach used to increase the amount of enzyme on the electrode to allow detection of non-turnover signals (Appendix 1). Such modification was carried out, but the signals were not observed disallowing the calculation of electrolytic coverage, Γ .

Two traces are shown in Figure 44, one corresponding to an experiment where TCE (red) was introduced by injection and another to the injection of PCE (black) (both to a final concentration of 0.8 mM). The same onset potential was observed for each, which means that the redox potential for each substrate being processed at the active site had no effect on the onset potential. Hexter *et al*²⁹ modelled the bias that is displayed by an enzyme in terms of the potential difference that exists between the Fe–S cluster that serve the point of entry for electron for reduction and the redox potential of the occurring at the active site. The profiles seen in Figure 44 do indicate similar *bias* of the enzyme towards the two substrates, PCE and TCE, by the similarity in the shape of their normalized profiles. Given the redox-potential of the Fe-S cluster forming the gateway to the entering electron is the same and the reported redox potentials of PCE to TCE and

TCE to *cis*-DCE are of +0.58 V and +0.54 V (at pH 7) respectively, the similarity in electrochemical profiles is not surprising. Furthermore, based on this, the decision was taken to pursue further work with TCE which is less harmful than PCE but would provide the same information.

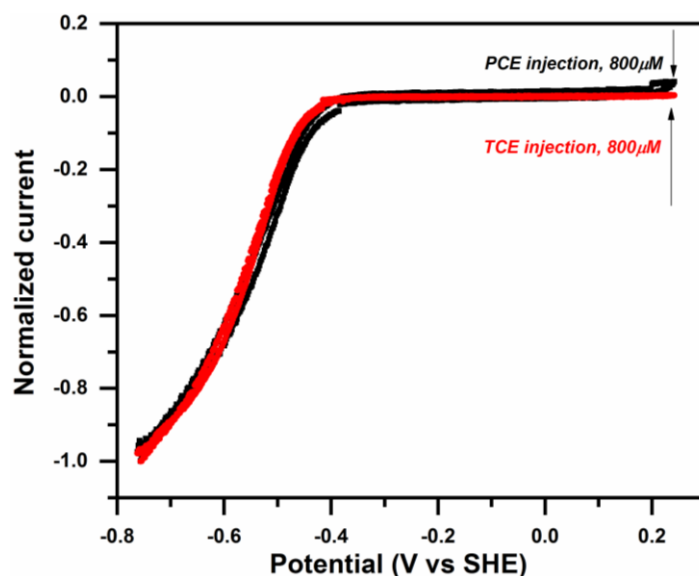


Figure 44: Electrocatalytic profiles of PceA dehalogenase with PCE and TCE. The electrochemical cell was filled with 100 mM TRIS+ 4mM (NH₄)₂SO₄ as electrolytic solution. PCE or TCE was introduced by injection at the positive end of the potential sweep to yield final concentrations of 0.8 mM in the electrolytic solution. The profiles with PCE and TCE were similar and based upon the fact that among them TCE is less toxic, it was chosen as substrate for the PFE experiments. Similarity in profiles indicate the same mechanism of processing of the two substrates by the enzymes. Experimental conditions: T = 25°C, pH = 7 and rotation rate = 1000 rpm.

4.2.2 Investigation into Buffer Interactions by Cyclic Voltammetry

It has been reported that the presence of an ammonium salt enhances the activity of the PceA dehalogenase.⁸³ Two separate experiments, identically carried out with an enzyme-coated PGE electrode with TCE injected to give the same final cell concentration, were carried out both in the presence and absence of ammonium sulfate ((NH₄)₂SO₄) in TRIS buffer.

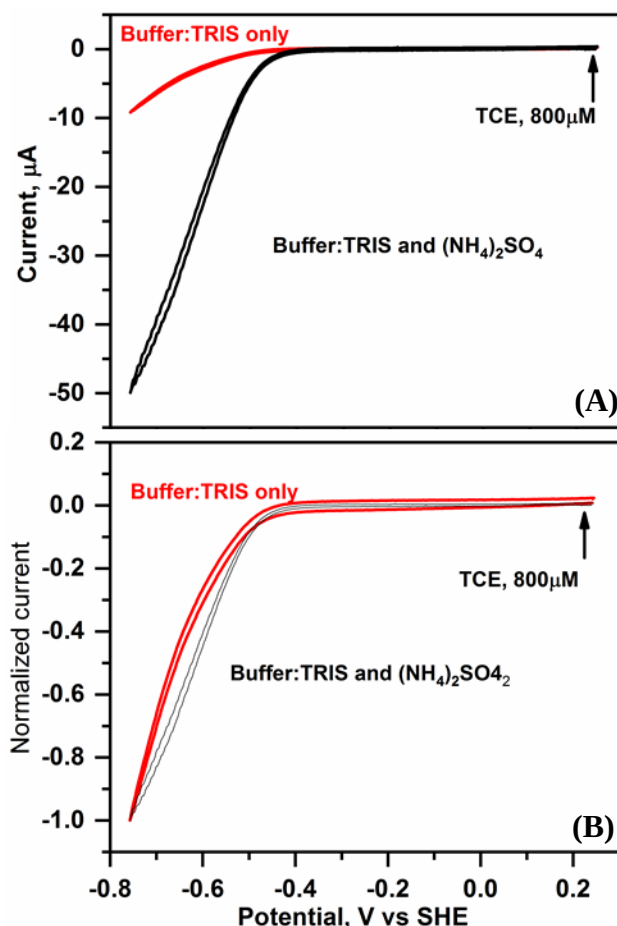


Figure 45: Effects of $(\text{NH}_4)_2\text{SO}_4$ in the electrolytic solution on PceA-catalysed TCE reduction to *cis*-DCE. (A) PGE electrode with the same film of PceA dehalogenase was transferred between electrochemical cells, one with electrolytic solution (100 mM TRIS; pH=7) to which 4 mM $(\text{NH}_4)_2\text{SO}_4$ has been added and another without the $(\text{NH}_4)_2\text{SO}_4$. It is seen that activity in TRIS only buffer is lower in agreement with reports in literature.⁸³ (B) The data in (A) is normalized and shown here to allow direct comparison. It is seen that the onset potential is unchanged between the two cases but for TRIS-only buffer the rate of reaction is lower despite the same driving overpotential being applied. Experimental conditions: initial TCE concentration= 0.8 mM, T = 25°C and rotation rate = 1000 rpm.

The same film of enzyme between the experiments was used to allow a direct comparison. Although Figure 45A (raw data) does show that the activity of the enzyme in pH=7, 100 mM TRIS buffer is lower (cathodic current by the red trace is lower) than that in 100 mM TRIS with $(\text{NH}_4)_2\text{SO}_4$ added to it, no change was observed in the onset potential of the voltammograms upon normalization of the raw data (Figure 45B). Thus

the presence of $(\text{NH}_4)_2\text{SO}_4$ in the buffer makes no difference to the mechanism at the active site or the reduction potential of the Fe–S cluster that serve as the point of entry for electrons for reduction.

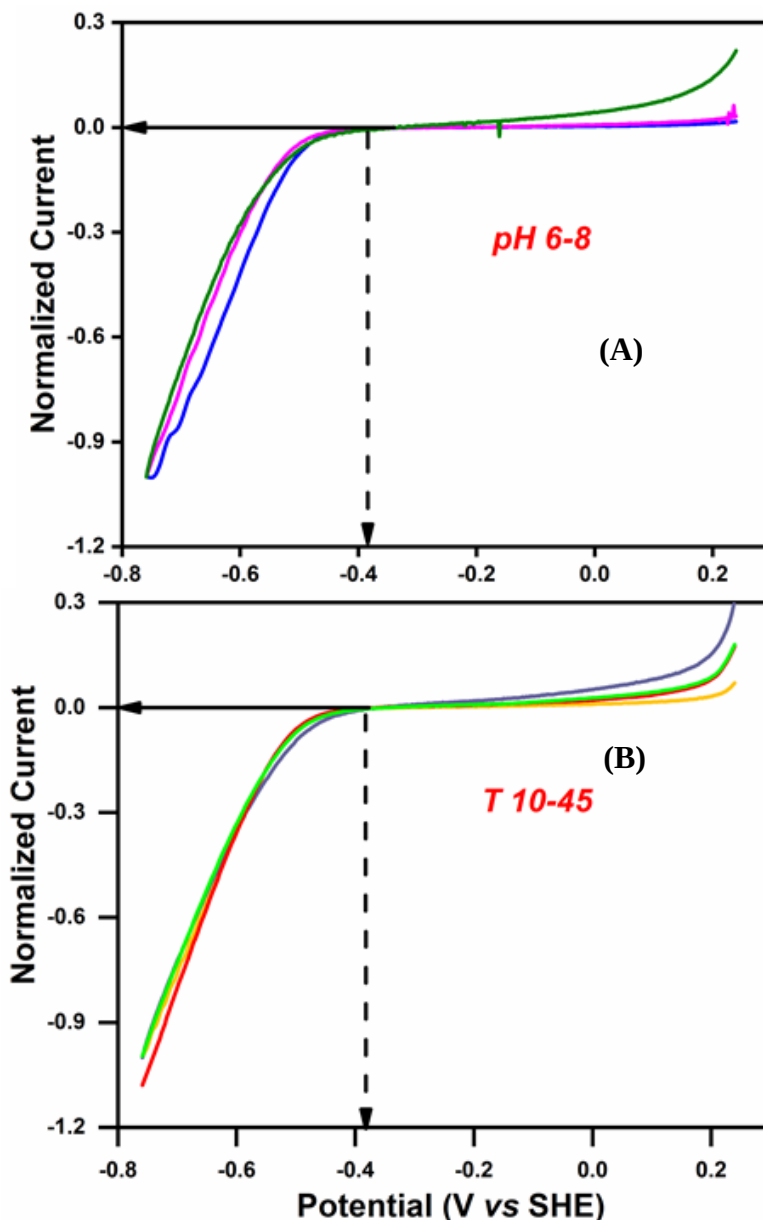


Figure 46: The effects of pH and temperature of the electrolytic solution upon PceA-catalyzed TCE reduction. (A) In the pH range (pH 6-8) and (B) temperature range (10 – 45 °C) tested, similar electrochemical profiles were obtained indicating that similar mechanism occurs throughout. The absence of shift in the onset potential with pH is indicative that proton (H^+) does not play a part in the rate-determining step of the redox reaction occurring at the active site. Experimental conditions if not otherwise mentioned: pH = 7, T = 25°C and rotation rate= 1000 rpm.

pH and temperature effects

Effects of pH and temperature of the electrolytic cell was investigated by CVs. The pH is known to change the onset potential when a proton (H^+) is part of the rate-determining step but temperature usually does not affect the onset potential, provided the mechanism remains the same across the temperature range being tested. A PceA-coated PGE electrode was transferred between cell solutions of different pH and CVs taken while potential was swept from -1 to 0 V vs SCE and back. The results of the scans at different pH (calibrated vs SHE) were then normalized and is shown in Figure 46A. The normalized results of similarly conducted experiments but with changing temperature (in this case, 10 – 45 °C). are shown in Figure 46B. The effects of pH and temperature on the reaction of PceA dehalogenase with TCE seems to be the same across the values tested indicating that H^+ does *not* play a role the rate-determining step and the mechanism of TCE processing by PceA dehalogenase is the same across the temperature range tested.

4.2.3 PFE of the Corrinoid Cofactor

The PceA dehalogenase contains norpseudo B-12 corrinoid cofactor optimally positioned in the active site for carrying out reduction of the substrate. It has been reported that the isolated corrinoid cofactor is capable of carrying out reduction of PCE and TCE. The corrinoid cofactor was provided by our collaborators at University of Jena, Germany where it was isolated as described previously.⁸⁶ A small quantity (1-2 μ l of corrinoid sample of concentration 500 nM) were spotted onto a PGE electrode and a potential range from -1 to 0 V vs SCE applied *via* potentiostat. Figure 47 shows the cyclic voltammogram resulting from the adsorption of the cofactor on the PGE electrode.

A set of redox peaks (cathodic and anodic) whose average value calculated to be -0.330 V vs SHE were observed. Redox peaks are reflective of a redox active centre and it may be assumed that the cobalt in the norpseudovitamin B12 corrinoid cofactor is the relevant centre. Thus, at -0.33 V, the cobalt undergoes a change in its oxidation state. This value is much more positive and is more comparable with that reported for the cofactor buried within the protein (-0.380 V).⁸⁶

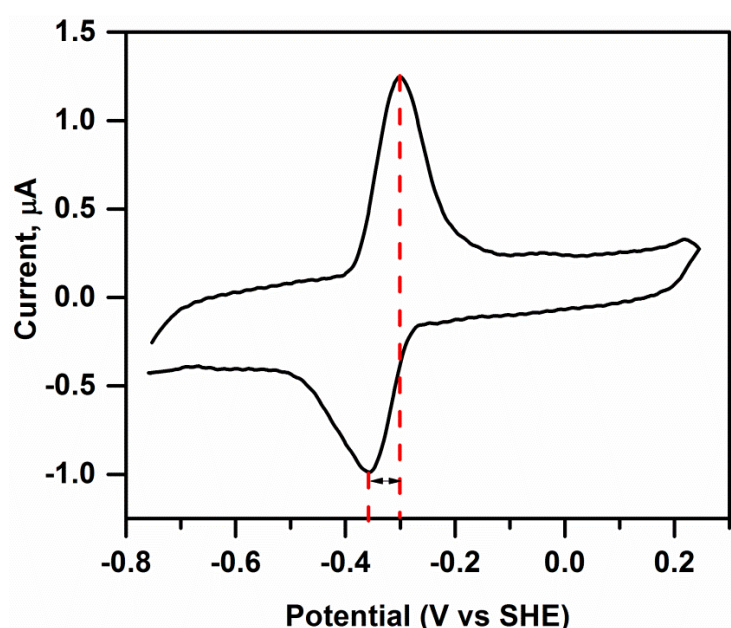


Figure 47: The electrochemical profile of corrinoid cofactor on stationary PGE electrode. Upon spotting the isolated corrinoid cofactor onto PGE electrode which was subsequently put in 100 mM TRIS solution with 4 mM $(\text{NH}_4)_2\text{SO}_4$ ($\text{pH} = 7$) in a three-electrode electrochemical cell, two peaks are observed corresponding to the anodic and cathodic electron transfer at the redox active cobalt of the cofactor when substrate (TCE) is absent. The average of the values of the peaks (red dashes) is -0.330 V. The peaks are absent when TCE is present in the electrolytic solution.

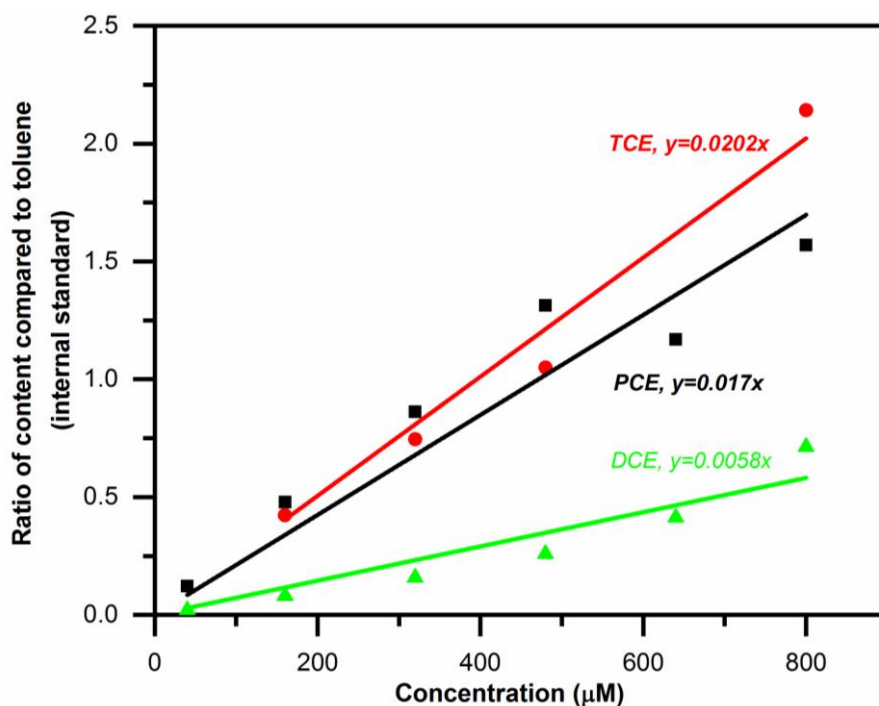


Figure 48: Calibration curves of PCE, TCE and *cis*-DCE as carried out by GC-MS. The substrates and product were dissolved in ethanol and then in 100 mM TRIS and 4 mM (NH₄)₂SO₄ buffer (pH=7) to make up to volume. Toluene was used as internal standard for autosampling by GC-MS: the equipment was run by Dr. James Wickens (CRL, University of Oxford, UK). The peak areas from the GC-MS spectra were used to create the calibration curves.

4.2.4 Detection of TCE, PCE and *cis*-DCE by GC-MS

Detection of TCE, PCE and *cis*-DCE is possible by gas chromatography–mass spectroscopy (GC-MS). In this technique, various components of a mixture may be separated by a chromatography based on affinity differences with the column material. As these components exit the column, they are subjected to ionization and a detector to yield mass spectra. The spectra are compared to a library of spectra from various compounds to serve as a product identification device. Once identified, the process can be repeated with various standards to yield calibration curves as shown in Figure 48. The standards were submitted to Dr. James Wickens in CRL to run the GC-MS

equipment. The results were subsequently analysed for understanding the effects of pH and temperature on PceA- catalysed TCE to *cis*-DCE conversion at the electrode.

4.2.5 Chronoamperometry: Stability and activity measurements

Chronoamperometry highlights the stability of the PFE set-up being used and the extent of ‘film loss’ that occurs during an experiment. Figure 49 shows an arrangement where an initial injection of TCE (pt. A: 800 μ M) in a mixed buffer of TRIS (100mM) and $(\text{NH}_4)_2\text{SO}_4$ (4mM) was followed over time until the current recorded was $\sim 2.5 \mu\text{A}$. Buffer exchange was carried out (pt. B) whereby the electrolytic solution was replaced by a fresh buffer solution (same volume as before). A drop to zero current indicated the successful exchange of buffer such that no substrate remained (pt. C). A fresh injection of TCE (pt. D: 800 μ M) resulted in an increase in current to a level greater than it was at point B because of the replenished substrate but still not at a level equal to that achieved at point A due to film loss which must have occurred between points A to D.

An extended chronoamperometry experiment with repeated buffer exchanges and injection of same amount of substrate will yield in further points similar to D, the collection of which may be used to calculate film loss similar to that shown in Appendix 3. Samples from points A and D were subjected to analysis by GC-MS and the calibration above (Figure 48) was used to confirm the substrate concentration at 800 μ M. This experiment additionally displays the stability of the arrangement over 6000 seconds with 33% loss of current signal.

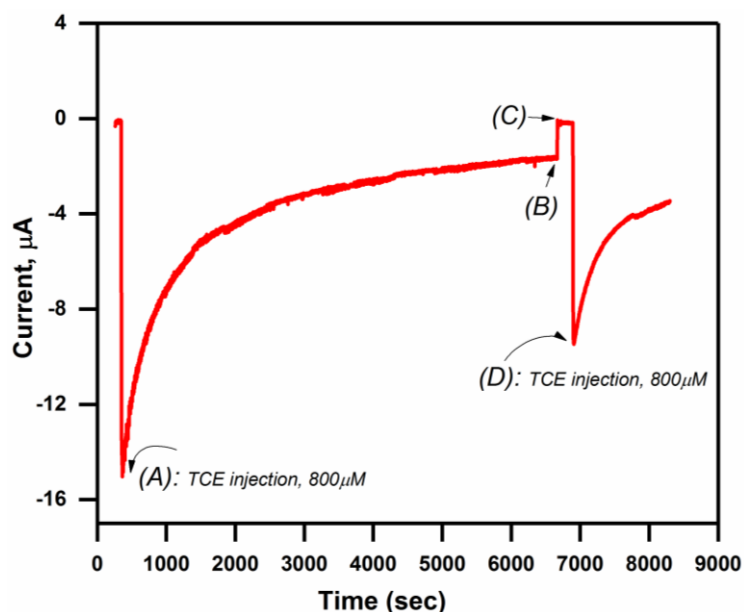


Figure 49: Chronoamperometry at -1 V vs SCE with PceA dehalogenase on PGE electrode with 100mM TRIS and 4mM $(\text{NH}_4)_2\text{SO}_4$. Initial injection of 0.8 mM TCE resulted in the increased current seen at pt A and withdrawal of a sample confirmed 0.8 mM concentration in the electrolytic cell. The subsequent fall in cathodic current can be attributed to film loss and loss in substrate from the cell solution. A buffer exchange at pt B resulted in a complete fall in current to pt. C. A fresh injection of TCE (0.8mM) results in pt.D whose concentration was also confirmed by GC-MS to be 0.8mM. However, the current is less than that at pt A and the difference can be used to calculate film loss similar to the procedure given in Appendix 3. Additionally, this experiment displays the stability of the experimental set-up as the catalytic current is obtained even after 6000 s of running the chronoamperometry. This time-frame is more than adequate for most PFE experiments. Experimental conditions: $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

A control experiment (not shown) was carried out in the absence of the enzyme to track the loss of the substrate from the cell over time (due to its volatility and therefore its evaporation into the headspace). After accounting for these losses (film loss and substrate loss), the resulting chronoamperometry trace should enable the tracking of the moles of substrate consumed with time. Validity of this hypothesis was provided by comparison of the calibration curves obtained by GC-MS (Section 4.2.4) and the chronoamperometry trace obtained at pH 7 and initial TCE concentration of 800 μM .

Both were in agreement with each other and were thus used in investigating the effects of pH and temperature by chronoamperometry as shown in Figure 50.

Panel A of Figure 50 shows a chronoamperometry experiment during which, pH was varied in order to investigate its effect on TCE consumption by PceA dehalogenase. It should be noted that the values obtained would not be absolute rates but comparative in nature (in this case pH 7 served as the baseline value) and that injections of TCE at pH 7 were carried out before the pH 9 and pH 8 experiments (not shown) to provide points with which to calculate film loss. Substrate loss correction data was provided by the control experiment as mentioned above.

An injection of TCE (0.8 mM) results in a spike in the cathodic current which falls at different rates depending upon the pH of the electrolytic solution. At pH 8, the slope of the fall is higher than that observed for pH 7 and pH 9 which indicates that pH 8 is the optimum pH of TCE consumption by PceA dehalogenase. Table 4 shows the rates after the raw chronoamperometry data is adjusted for film loss and substrate loss and then normalized with that at pH=7 being the baseline. The values confirm what can visually be seen in the chronoamperometry that TCE consumption by PceA dehalogenase is the highest in pH 8. This is also in agreement with values mentioned in literature.

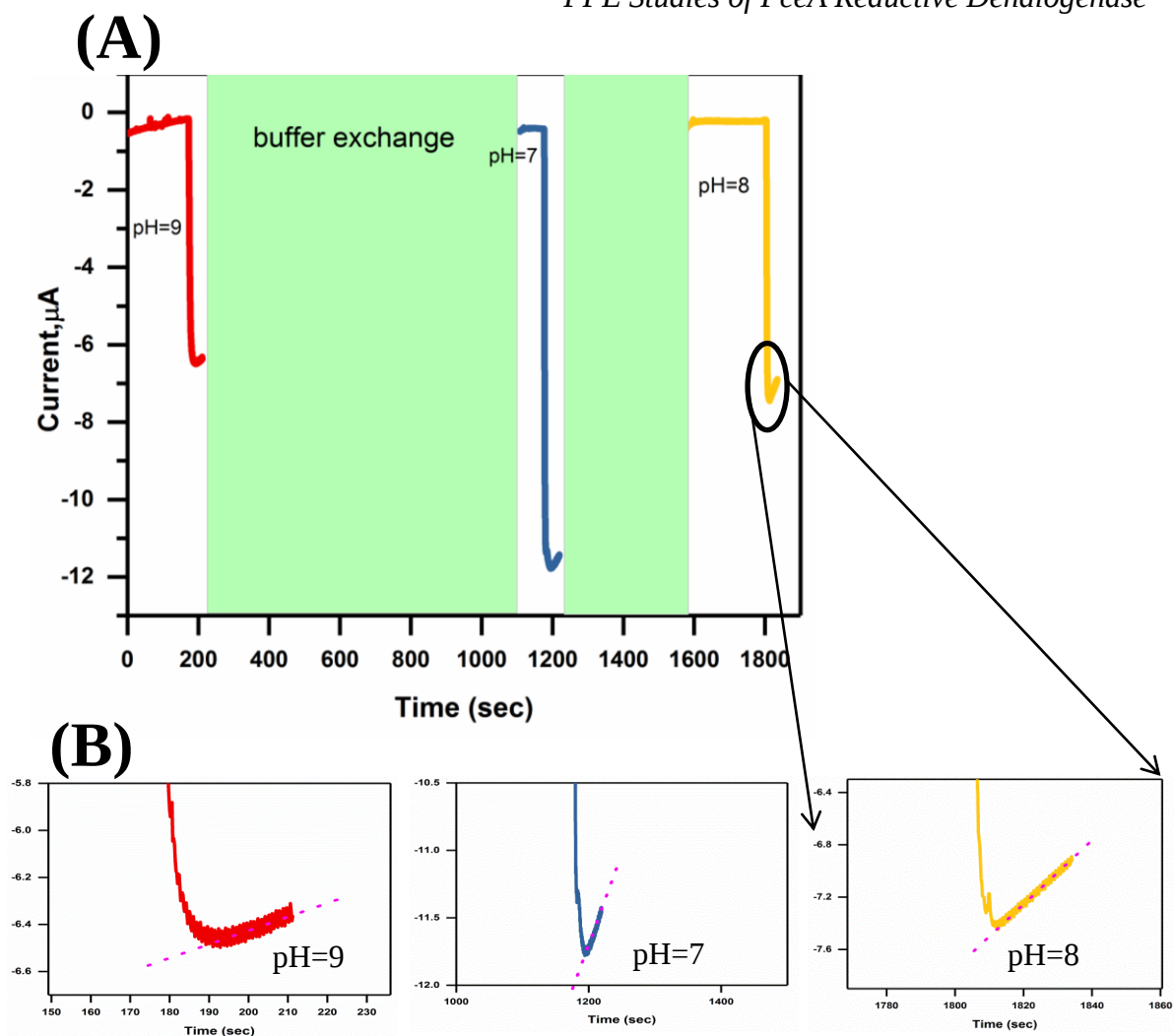


Figure 50: Panel A shows chronoamperometry to measure rate of TCE consumption by PceA dehalogenase in 100 mM TRIS and 4 mM $(\text{NH}_4)_2\text{SO}_4$ solutions at different pHs. The chronoamperometry trace is obtained by transfer of PceA dehalogenase-coated PGE electrode between the different solutions. Not shown is the chronoamperometry trace at pH 7 before and after pH 9 experiments and after the pH 8 experiment. As discussed in Figure 49, the three points obtained immediately following injection at pH 7 were used to calculate film loss and subsequently utilized for correction purpose of pH 7-9 readings. The recalibrated slopes indicate the rates of TCE consumption at different pH which is tabulated in Table 4. Experimental conditions: $T = 25^\circ\text{C}$ and rotation rate = 1000 rpm.

Table 4: Rates of TCE consumption by PceA dehalogenase at different pH (normalized with that observed at pH 7). The rates are calculated from the slopes of chronoamperometry experiments to which film loss and substrate loss correction has been applied.

<u>pH</u>	<u>Experimental rate normalized with that at pH 7</u>
7	1.000
8	1.561
9	0.907

A similar experiment was carried out with temperature variation, the results of which are tabulated below in Table 5. As may be expected, upto 35°C, the rates of TCE consumption increases with temperature.

Table 5: Rates of TCE consumption by PceA dehalogenase at different temperatures (normalized with that observed at T= 25°C). The rates are calculated from the slopes of chronoamperometry experiments to which film loss and substrate loss correction has been applied.

<u>Temp.(°C)</u>	<u>Experimental rate normalized with that at T= 25°C</u>
10	0.852
25	1
35	1.249

The data for the temperature variation was also used to calculate activation enthalpy, $\Delta H^\ddagger$ (Appendix 8) by plotting $\ln(k/T)$ vs $\frac{1}{T}$. A value of 23.2 ± 3.70 kcal mol⁻¹ was obtained for the activation enthalpy at -0.76 V vs SHE (the potential at which chronoamperometry was carried out). This is similar to values obtained for the Co-C bond dissociation energies in co-enzymes and organocobalt compounds including Schiff base and dimethylglyoxime compounds.¹⁵⁷

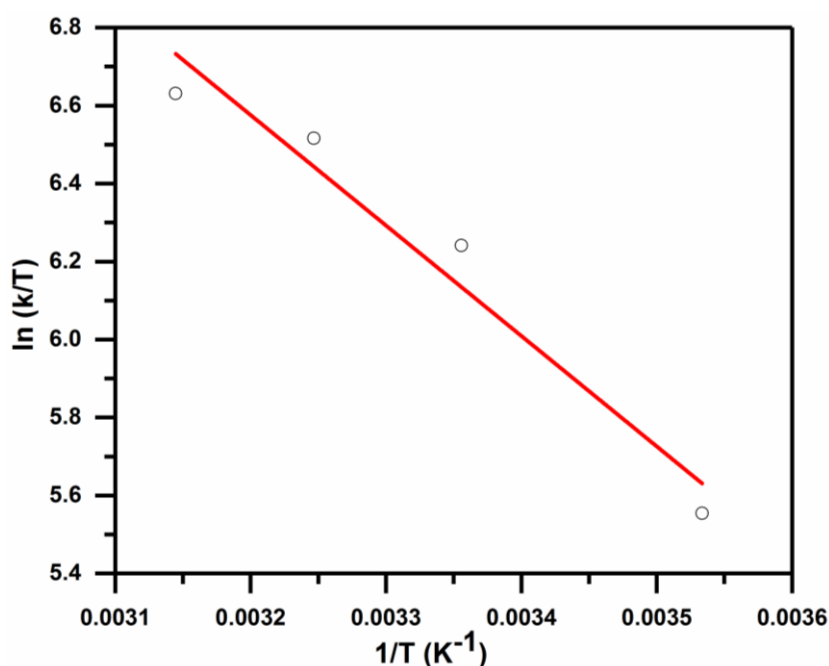


Figure 51: Plot of $\ln(k/T)$ vs $\frac{1}{T}$. The straight line obtained by plotting $\ln(k/T)$ vs $\frac{1}{T}$ gives a slope equivalent to $-\Delta H^\ddagger / R$ according to Eyring equation. A value of 23.2 ± 3.70 kcal mol⁻¹ was obtained for the activation enthalpy at -0.76 V vs SHE. This is similar to the values obtained from investigation into the cobalt-carbon bond dissociation energy of coenzyme Halpern *et al.*¹⁵⁷ Experimental conditions: Buffer = 100 mM TRIS with 4mM (NH₄)₂SO₄, T = 25°C and rotation rate = 1000 rpm.

4.2.6 PFE of PceA Dehalogenase with Alternate Substrates

The PceA dehalogenase is known to be able to process several substrates other than PCE and TCE. *Cis*-dibromoethylene (DBE) and 2,3-dichlorophenol (DCP) were chosen as subjects of study to compare with TCE. Their normalized reductive profiles are shown in Figure 52. The onset potentials for the two substrates are more negative than that of TCE. Given the onset potential is a reflection of the potential of the electrocatalytic ‘control centre’, the shift reflects a change in the potential of the centre to more negative values. From earlier experiments, it can be inferred that the cobalt in the corrinoid cofactor is the relevant control centre. A shift to more negative values

could reflect the stabilization of the more oxidized form of the cobalt (either Co^{II} or Co^{III} ; see Section 1.6.4) or the de-stabilization of the transition state: in the first instance, more driving force is required to access the catalytically active Co^{I} state or in the second case, access to the transition state invoke higher activation energy.

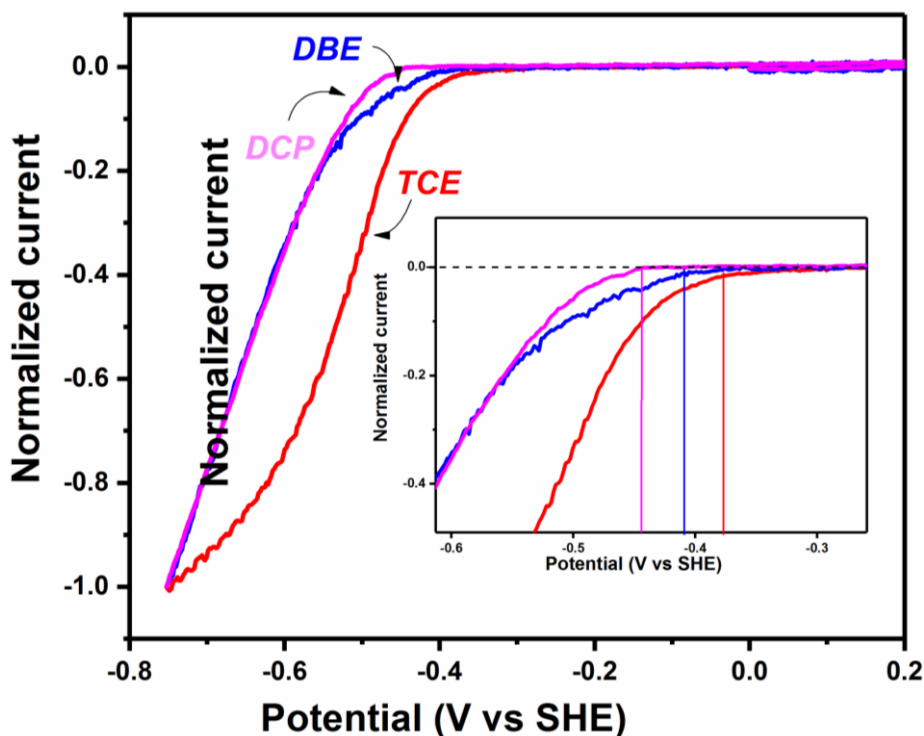


Figure 52: The normalized electrocatalytic profiles of substrates *cis*-dibromoethylene (DBE) and 2,3-dichlorophenol (DCP) compared to that of TCE. All substrates present in the system are at a concentration of 0.8 mM in the electrolytic solution– the onset potential of TCE is as before at -0.38V but those of DCP and DBE are obtained at more negative values. This is indicative of the stabilization of the $\text{Co}^{\text{II}}/\text{Co}^{\text{III}}$ state of the active site or the destabilization of the transition state which forms when DCP and DBE are present in the system instead of TCE as substrate. Experimental conditions: Buffer =100 mM TRIS with 4 mM $(\text{NH}_4)_2\text{SO}_4$, $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

4.2.7 PFE of PceA Dehalogenase with Inhibitors

Inhibition by KCN: There are many inhibitors for PceA dehalogenase –among them KCN has been reported widely as a potent inhibitor.¹⁵⁵ Thus cyclic voltammograms with and without KCN were studied. The black trace in Figure 53 is a voltammogram

obtained when an PceA-coated PGE electrode is immersed in 100 mM TRIS buffer solution (to which 4 mM $(\text{NH}_4)_2\text{SO}_4$ had been added) and the substrate, TCE, is injected at the positive end of the voltage sweep (final concentration in cell = 800 μM). The blue trace is of a similar experiment to which, additionally, KCN was injected at the positive end of the voltage sweep to give 10 mM final concentration. It is seen that the onset potential is shifted to more negative values by ~ 70 mV. The two voltammograms were normalized to allow comparison.

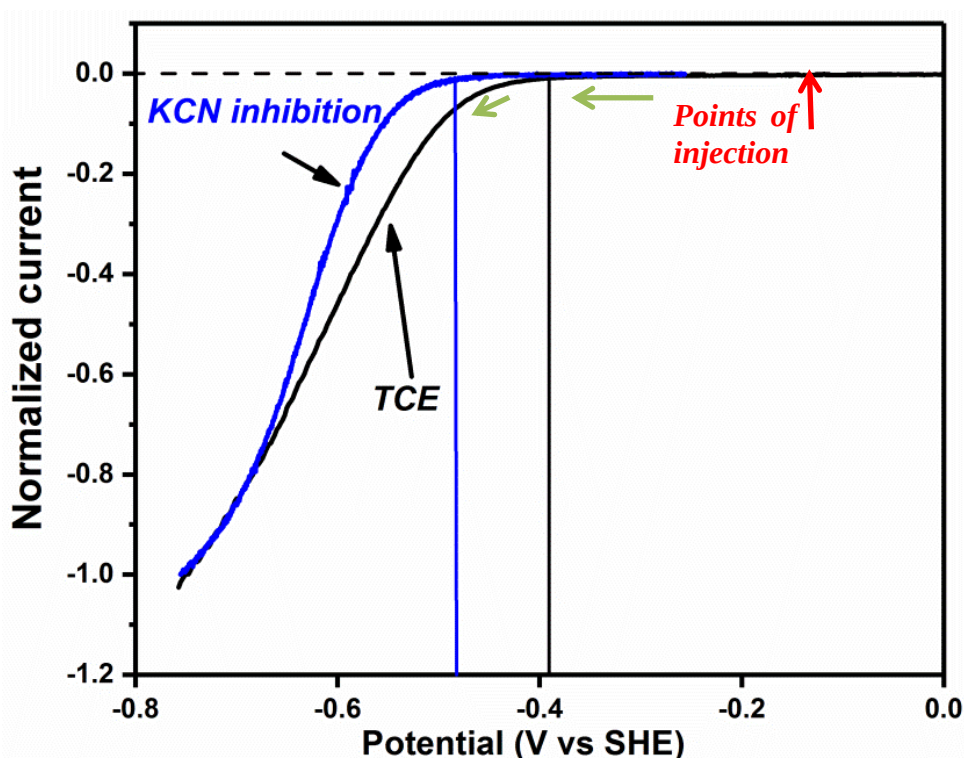


Figure 53: The shift in onset potential upon addition of KCN, an inhibitor, to a PceA-adsorbed PGE electrode. The black trace was obtained by a voltage sweep in which 0.8mM TCE was added at the positive potential end (indicated by red arrow) before sweep to the negative potential began. Green arrows give the direction of voltage sweep. The blue trace was obtained in a similar experiment to which KCN was also added at the positive end of the voltage sweep to yield 10 mM final concentration. The electrolytic solution is a 100 mM TRIS buffer solution to which 4 mM $(\text{NH}_4)_2\text{SO}_4$ have been added. The onset potential of the blue trace is seen to be shifted to more negative values by ~ 70 mV. Experimental conditions: $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

The shift to more negative potential has been observed previously in the case of enzyme catalysing the reduction of DBE and DCP. Figure 54 shows the difference between an electrochemical system in which TCE has been injected followed by injection of DBE (a substrate) (Figure 54A) and one in which TCE is inhibited with KCN as shown in Figure 54B. Two differences are immediately evident: 1. There is a drop in current at the point of KCN injection (green trace in Figure 54B) but no such drop is seen on injection of DBE (Figure 54A) and 2. At the most negative end of the voltage sweep, the CV for KCN injection shows a decrease in current between the 1st and 2nd scan while the reverse is true in case of DBE injection. While the applied potential is more negative than -0.48 V, even though KCN is in the system, the catalytic activity of the enzyme is evident through the observation of a reduction current.

To demonstrate the potential dependency of the KCN inhibition, two potentials were chosen to study the effect as shown in Figure 55. At -0.68 V, an injection of KCN to a final concentration of 10 mM, brings about an immediate inhibition to result in a loss of activity by $\sim 23\%$ (Figure 55A) while at -0.50 V (Figure 55B), an injection of KCN to a final concentration of 10 mM results in an 87.5 % fall in activity. Since the drops in activities of the PceA-catalysed TCE reduction is varied even though the same amount of KCN is used, it may be inferred that the inhibition is strongly potential dependent. Furthermore, it indicates that the lowering of potential at the PGE electrode in PFE experiments brings about dissociation of KCN which was attempted in previous study with mediator but was not successful.¹⁵⁵ Between -0.480 V and -0.380 V complete inhibition by KCN occurs.

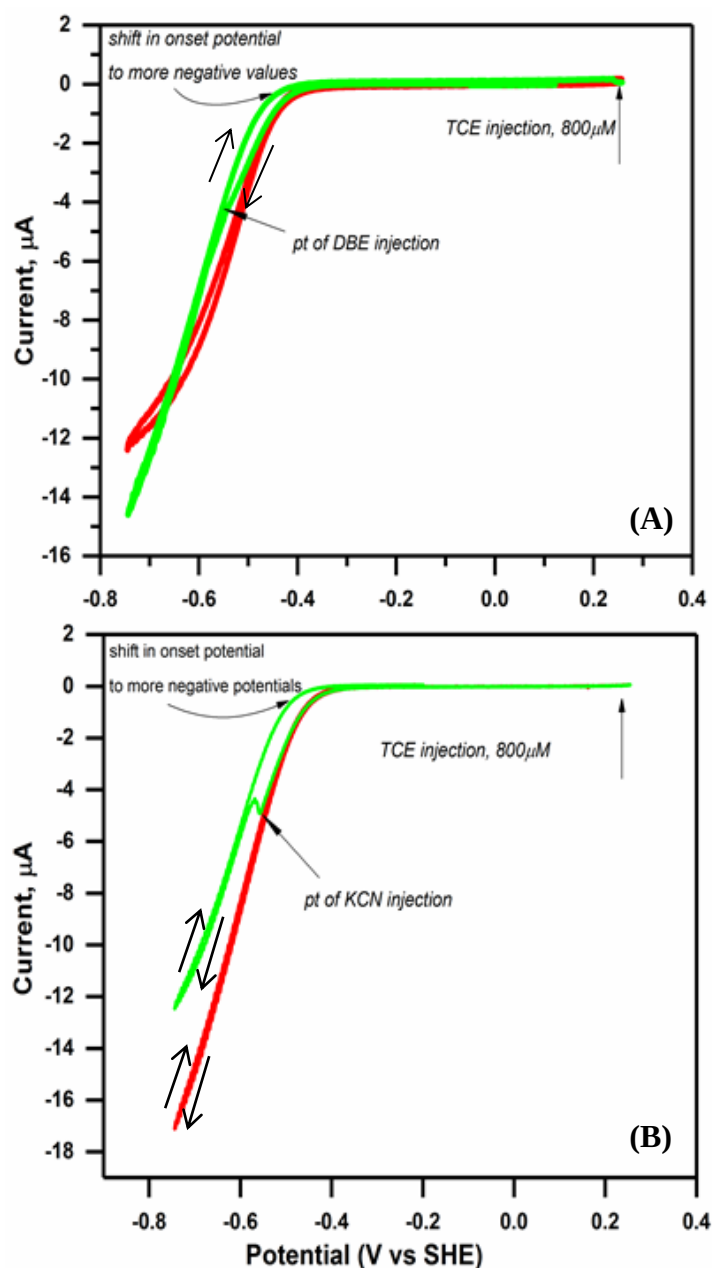


Figure 54: The effects of injection of DBE (a substrate) and KCN (an inhibitor) in PceA dehalogenase-TCE system. Panel A shows the 1st scan in red where a reductive current is seen when TCE (0.8 mM) is added to an electrochemical cell in which the PceA-adsorbed PGE electrode serves as the working electrode. The green trace is the 2nd scan in which DBE is injected at -0.58 V. The value of the current at the more negative end is bigger than that with the 1st scan showing that the increased concentration of the substrate has increased the value of the current generated. Panel B shows a similar set-up in which injection with KCN (10 mM) is carried out and results in fall in current at the point of injection as well as at the most negative end. These differences arise between Panel A and Panel B because KCN is an inhibitor and not a substrate like DBE. Experimental conditions: 100mM TRIS buffer solution to which 4 mM $(\text{NH}_4)_2\text{SO}_4$ has been added, $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

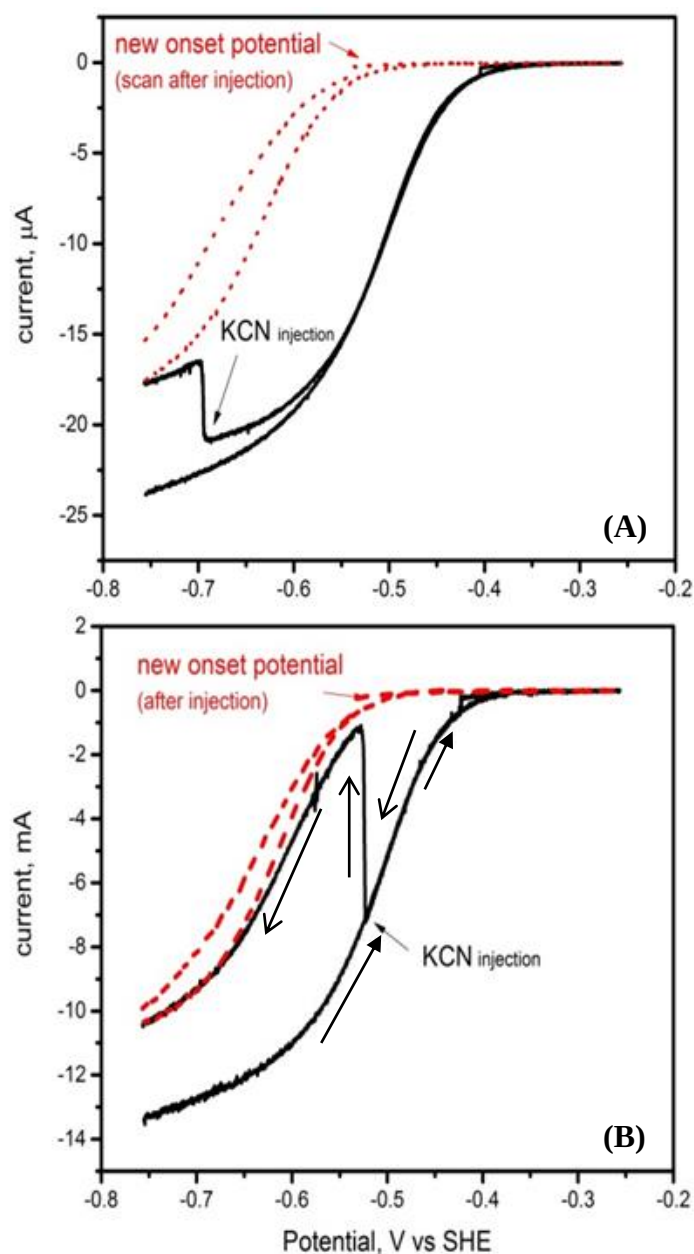


Figure 55: Inhibition by KCN of Pce-A catalyzed TCE reduction at different potentials. The effect of potential on the amount of inhibition that 10mM KCN bring about was investigated by comparison at -0.68V and -0.50V. The black traces in both Panel (A) and (B) are the 1st scans of their respective experiments and begins at the most negative end and sweeps towards the most positive and back. Upon the reverse scan, 10mM of KCN is introduced into the electrolytic solution at the potentials -0.68V in Panel A and at -0.50V in Panel B. 23% fall in activity was observed in Panel A while 87.5% fall took place in Panel B. Thus a more negative potential brings about greater inhibition. Experimental conditions: 100mM TRIS buffer solution to which 4mM $(\text{NH}_4)_2\text{SO}_4$ has been added, TCE conc.= 0.8mM, $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

An investigation into the binding potential was carried out by chronoamperometry experiment, the result of which is shown in Figure 56. The chronoamperometry experiment was carried out by alternating between a high potential (at which there is no catalytic current however binding of KCN may still occur) and a low potential at which KCN should unbind if already bound or a reductive current observed if no KCN bound during the previous step. As expected, at 0.24 mV vs SHE no activity is observed because the dehalogenase is only capable of carrying out reduction at potentials above -0.38 V.

If no KCN were present in the system at all then this switch in potential from one at which the enzyme cannot catalyse the reaction to one at which it can, an increase in current would be observed. To note is the fact that upon the switch of potential to -0.56 mV, the current immediately begins to increase (i.e. become more negative). It is with this phenomenon (KCN unbinding) that we may assume at 0.240 mV, KCN has bound to the system.

At 140 mV and 40 mV again no catalytic activity is observed and the values of the initial reductive current on switching to -0.560 mV, are increasingly higher and the rates of unbinding (given by the slope) are increasingly lower. On switching from -60 mV to -560 mV, the current increases as expected due to now being at a potential at which the enzyme catalyses the reduction, however, no unbinding is observed as was the case for previous voltages. Thus it may be concluded that KCN did not bind at -60 mV and therefore its binding occurs at potentials in the range 40 mV to -60 mV vs SHE. Since -0.380 V is the onset potential and represents the conversion to the catalytically active

Co^{I} state, it may be inferred that between the 40 mV to -60 mV vs SHE, the cobalt at the active site undergoes $\text{Co}^{\text{II}}/\text{Co}^{\text{III}}$ conversion.

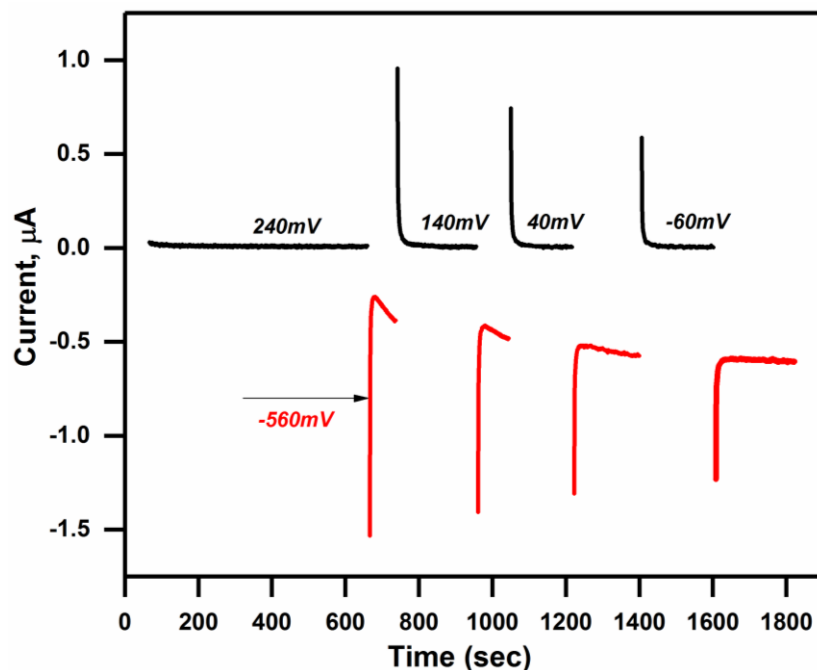


Figure 56: Investigation by chronoamperometry into the binding potential of KCN with the enzyme. A series of potential steps were applied (black traces) interspersed by poisoning at potentials of -0.56 V (red traces). At more positive potentials than -0.48 V, a potential will exist beyond which more positive values will cause binding of KCN to the enzyme and the dissociation will be observed when potential is brought down to -0.56 V. The slopes seen in the red traces indicate the recoveries that occur when dissociation occurs. Between applications of potentials from 0.24 V to -60 mV in steps of 100 mV, slope is seen except after application of -60 mV. This absence of the slope provides evidence that the binding potential exists between 40 mV and -60 mV vs SHE. Experimental conditions: 100 mM TRIS buffer solution to which 4 mM $(\text{NH}_4)_2\text{SO}_4$ has been added, TCE conc. = 0.8 mM, $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

Finally, the effect of the concentration of KCN in the electrolytic solution on the rate of inhibition was investigated *via* chronoamperometry, the results of which are plotted on Figure 57A. Figure 57B shows that the rate is directly proportional to the 1^{st} power of the concentration of the inhibitor.

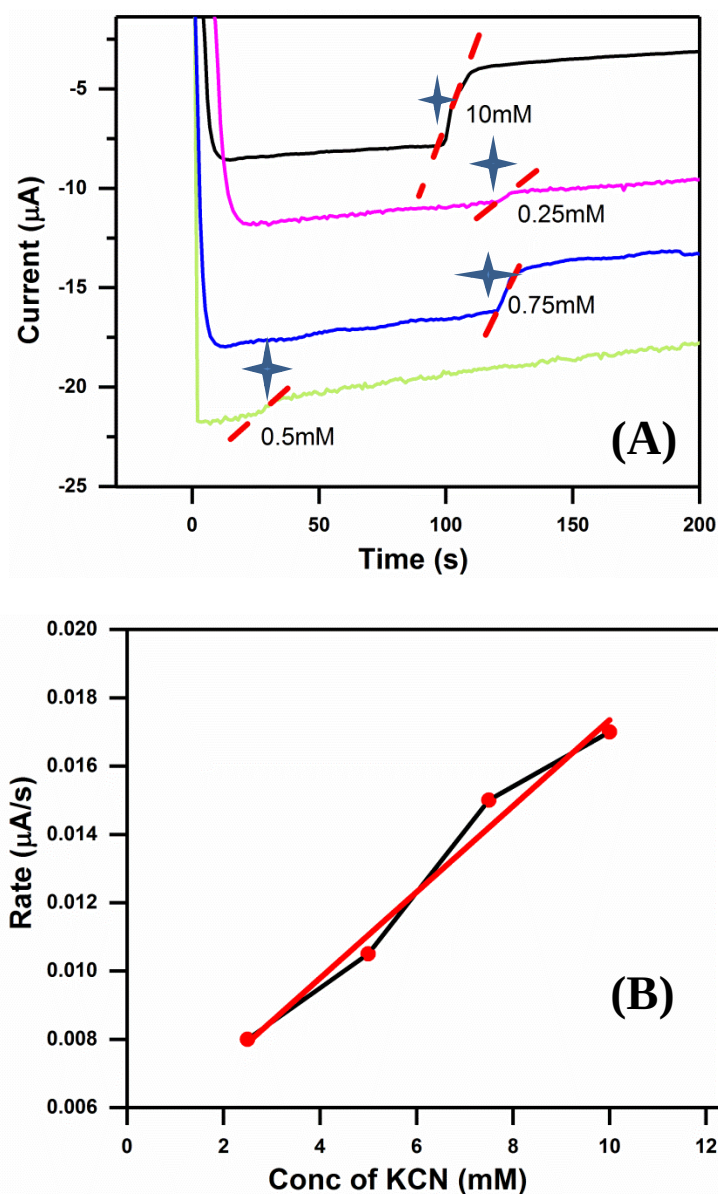


Figure 57: Chronoamperometry relating the concentration of KCN (inhibitor) with the rate of inhibition at -0.56 V vs SHE . (A) This panel shows the results of four sets of experiments done with the same film of enzyme where each set of experiment was preceded by buffer exchange to ensure the absence of KCN in the system. The constant slope until the point of injection (marked by a blue star) also confirmed that the buffer exchange was done successfully. Upon injection of different quantities of KCN to yield the final concentrations, the current dropped at different rates which are indicated by the different magnitude of the slopes. (B) The rates are given in terms of (i/t) in $\mu\text{A/s}$ as the exact coverage of the enzyme is not known – however, the slope does give the Δ (rates of inhibition)/ concentration in mM which provide information that the rate is directly proportional to the concentration in agreement with previous reports.¹⁵⁵ Experimental conditions: 100 mM TRIS buffer solution to which 4 mM $(\text{NH}_4)_2\text{SO}_4$ has been added, TCE conc.= 0.8 mM, $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

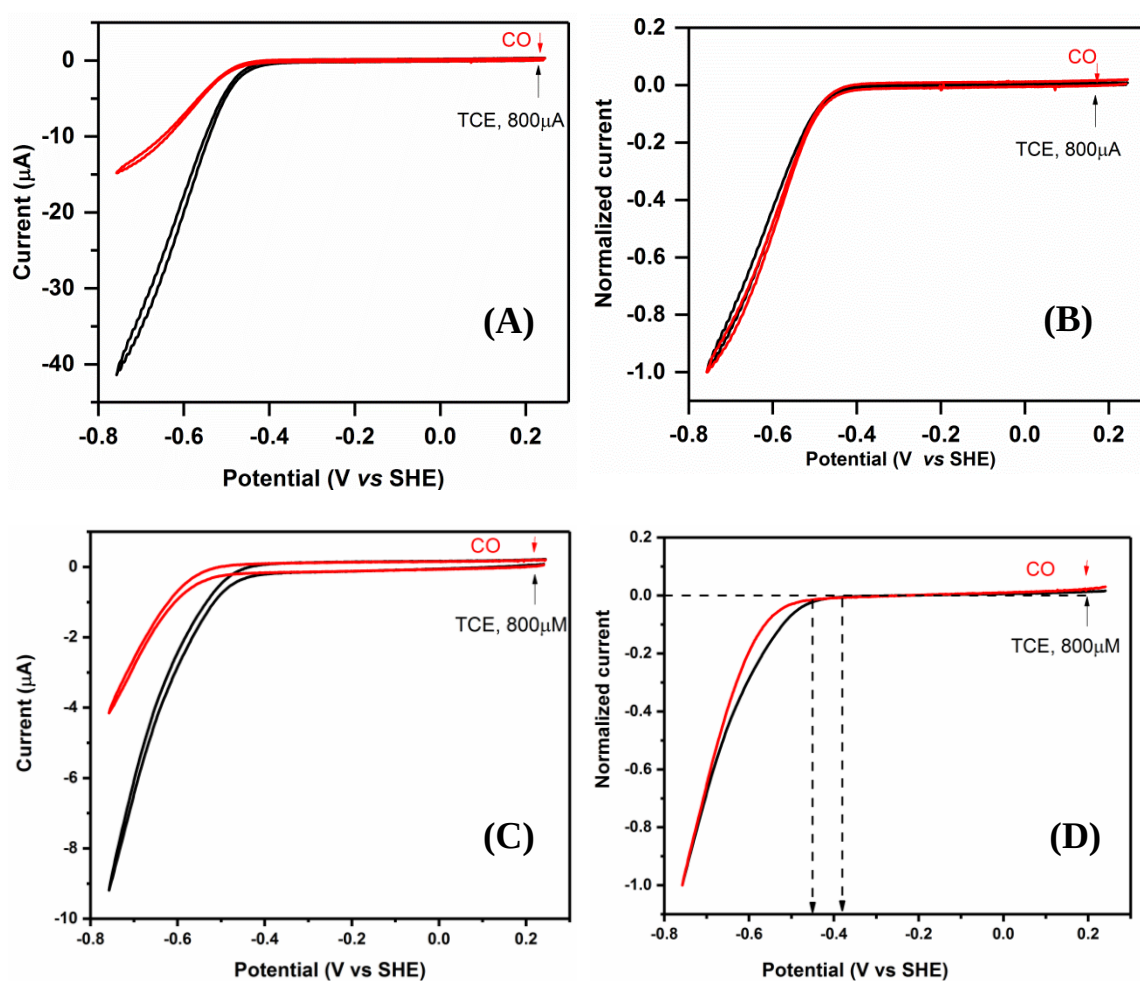


Figure 58: Inhibition of PceA-catalyzed TCE reduction by CO in TRIS buffer with and without added $(\text{NH}_4)_2\text{SO}_4$. The Pce-A dehalogenase is adsorbed onto PGE electrode to which TCE is added to yield a concentration of 0.8mM. The concentration of TRIS buffer is 100mM and when $(\text{NH}_4)_2\text{SO}_4$ is added, it was made to be 4mM. Panels (A) and (B) are a set of results carried out in TRIS buffer with added $(\text{NH}_4)_2\text{SO}_4$ in which (A) is the raw data while (B) is the corresponding normalized data. No shift in onset potential is observed as was observed in the case of KCN inhibition. Panels (C) and (D) are a set of results carried out in TRIS buffer without the $(\text{NH}_4)_2\text{SO}_4$ in which (C) is the raw data while (D) is the corresponding normalized data. A shift in potential is observed as opposed to that in $(\text{NH}_4)_2\text{SO}_4$ -added TRIS buffer. The shift is of $\sim 70\text{mV}$. Experimental conditions: $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

Inhibition by CO: CO and KCN are isoelectronic and while KCN is a known inhibitor of PceA dehalogenase, inhibition by CO has not yet been observed in any of the reported inhibition studies of PceA dehalogenase. Inhibition by CO was investigated by

injecting aliquots of CO-saturated solution into the electrolytic solution. Such a method was adopted rather than using CO gas in the headspace since this may remove the volatile substrates to levels too low to allow observation of catalytic current; in addition, by injecting CO as a solution the desired concentration is attained immediately without waiting for equilibration as in the case of using CO gas.

Two sets of voltammograms are compared in Figure 58. Panel (A) and (B) shows results of CO inhibition of PceA-catalyzed TCE reaction carried out in TRIS buffer with added $(\text{NH}_4)_2\text{SO}_4$ while Panel (C) and (D) show the results of comparable experiments in TRIS buffer without the salt. A typical experiment is where TCE solution is injected to a final concentration of 800 μM at the positive end of the voltage sweep and in the immediate scan afterwards CO is injected (again, at the positive end of the sweep) to a final concentration of 77 μM in the form of a CO-saturated solution. In Figure 58 A, the fall in activity cannot be assigned exclusively to CO inhibition as experience shows that loss may be due to volatility of substrate. Furthermore, it may also be due to CO displacing the substrate in solution or to dilution of the electrolytic solution. The corresponding normalized data (Figure 58B) highlights that there is no shift in onset potential in contrast to that observed for the case of KCN inhibition. However, the absence of $(\text{NH}_4)_2\text{SO}_4$ in the electrolytic solution₄ results in a very different profile. There is fall in activity in Panel C as seen in Figure 58 A but a shift in potential to more negative values is observed in the normalized figure in Figure 58D. The shift in potential highlights a $\sim 70\text{mV}$ shift upon injection of CO to a TCE solution. Thus, the effect of CO is more pronounced in the absence of $(\text{NH}_4)_2\text{SO}_4$ salt in the system and the magnitude of the shift in potential in the case of CO-inhibition in TRIS solution without

the $(\text{NH}_4)_2\text{SO}_4$ is the same as occurs in the case of KCN inhibition in TRIS buffer with added salt.

Figure 59 highlights the effect of a KCN inhibition on a solution of TCE (800 μM) with and without CO in 100mM TRIS buffer with 4mM $(\text{NH}_4)_2\text{SO}_4$. As it has been observed that an injection of CO-saturated solution causes a fall in current which cannot directly be attributed to CO inhibition (Figure 58 A and B), this experiment was conducted by first saturating the buffer solution in the electrolytic cell with CO by flowing into the headspace of the electrochemical cell for 0.5-1 hr. This was followed by injecting TCE to yield a final concentration of 0.8 mM and the resulting current is shown as a black trace (normalized version of raw data) in Figure 59. Then, KCN solution was injected to a concentration of 10 mM, to the electrolytic cell to yield the red trace (normalized version of raw data). It may be noted that a shift in onset potential is seen as before, but the magnitude of the is not as large as before. A similarly conducted experiment but without CO –saturation taking place prior to KCN injection yielded the blue trace which shows the $\sim 70\text{mV}$ shift to more negative potentials as seen before. The stabilization of the more oxidized form of the active site by KCN was proposed before as the cause for shift in potential to more negative values and it would seem that that effect is interfered by presence of CO. Together with the role of $(\text{NH}_4)_2\text{SO}_4$ as noted from Figure 58 in which its absence allowed CO-inhibition to occur, it may be proposed that $(\text{NH}_4)_2\text{SO}_4$ brings about conformational changes within the enzyme's active site which disallow the binding of CO to the active site. This binding site must also be relevant in the activity displayed by the enzyme such that binding with $(\text{NH}_4)_2\text{SO}_4$ enhances the activity of the enzyme – either by enhancing electron or proton transfer.

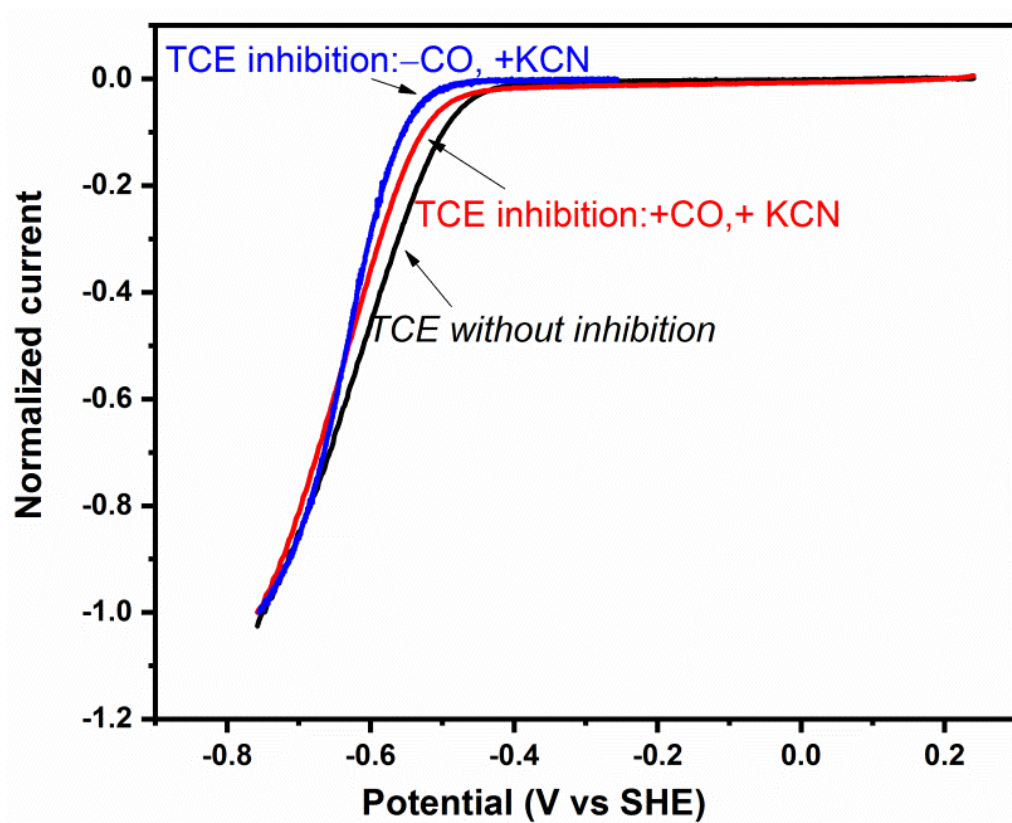


Figure 59: Inhibition profiles of PceA-catalysed TCE reduction with KCN with and without CO. The buffer solution in the electrolytic cell in which PceA-coated PGE electrode is dipped is first saturated with CO by flowing into the headspace of the electrochemical cell for 0.5-1 h. TCE is injected into the positive end of the CV to give a final concentration of 0.8mM. The black trace is thus obtained (normalized version of raw data). Then, KCN solution was injected to a concentration of 10 mM in the electrolytic cell to yield the red trace (normalized version of raw data). The blue trace is from another experiment in which the CO treatment was not carried out prior to KCN injection. It may be observed that, in the presence of CO, a shift in onset potential to more negative values is observed from that of uninhibited TCE curve (black trace), but the magnitude of the shift is not as large as without CO present (~ 30 mV *vs* ~ 70 mV). Experimental conditions: 100mM TRIS buffer solution to which 4mM $(\text{NH}_4)_2\text{SO}_4$ has been added, $T = 25^\circ\text{C}$, $\text{pH} = 7$ and rotation rate = 1000 rpm.

4.3 Conclusion and Perspectives

A reductive dehalogenase, PceA, from *Sulfurospirillum multivorans* was studied with PFE, the results of which are presented in this chapter. Previous research studies were not successful in studying the enzyme immobilized on an electrode without a mediator, however, as shown in this chapter, it was possible to study the enzyme on a Pyrolytic Graphite Edge (PGE) electrode directly. This opens a new avenue of research into the O₂-sensitive enzyme. No adsorbates or modification of the electrode was required to obtain a catalytic signal.

The nonpseudocorrinoid, part of the active site, was also observed as active on the PGE electrode with the peaks yielding a value of -0.33 V— a value which corresponds very closely to the reported value of -0.38 V for Co^I/Co^{II} conversion at the active site.⁸⁶ The catalytic profiles obtained upon PceA-catalyzed PCE/TCE reduction display an onset potential of -0.38 V which is the same as the potential reported of Co^I/Co^{II} conversion of the corrinoid cofactor at the active site.⁸⁶ This is significant as it denotes that electron transfer to the corrinoid cofactor is essential to initiate the reaction such that the active site can act as the reductant leading to the formation of a trichlorovinyl radical *via* an intermediate carbanion radical (Pathway Two as discussed in Section 1.6.4).

The shift in onset potential to more negative values upon KCN binding to PceA dehalogenase of the active site, however, would indicate the binding of KCN to the corrinoid of the active site. This binding was previously thought to be irreversible as titanium citrate was unable to provide the low reduction values that may be achieved with PFE. This supports that Co in the +3 state is indeed the state and site of binding of KCN. Our study shows that the binding occurs at potentials between 40mV and -60mV

vs SHE and that rate of the inhibition is proportional to the 1st power of KCN concentration. This supports the proposal that in the catalytic cycle, Co^I of the active site acts as a strong nucleophile and attacks the carbon backbone (Pathway One as discussed in Section 1.6.4) in contrast to the conclusion drawn earlier.

Carbon monoxide, isoelectronic to cyanide, has been described to bind to the cobalt of corrinoids as a complex ligand. Previously, CO (100% in the gas phase of the extracts and/or of the cuvettes) did not influence the dechlorination activity, but as described in this chapter, inhibition is visible when PGE-immobilized PceA dehalogenase is in TRIS buffer from which (NH₄)₂SO₄ is absent. Although the role of (NH₄)₂SO₄ could not be elucidated from PFE, the fact that its absence brought about CO inhibition may be of considerable interest. Also, in conjunction, CO prevents the binding/stabilization effect brought about by KCN but does so in the presence of (NH₄)₂SO₄. Given that KCN is thought to bind to the Co^{III} state of the enzyme and stabilize the Co^{II} state over the Co^I state, CO and (NH₄)₂SO₄ must bind to a residue that is near the cobalt. Further investigation, particularly crystallization, with either (NH₄)₂SO₄ and CO, may prove useful in gaining insight to the mechanism occurring at the active site with PCE and TCE. Since (NH₄)₂SO₄ enhances the catalytic performance of PceA dehalogenase, further investigation into its effect is warranted and maybe be achieved *via* CO-inhibition investigations as a new avenue of research.

The onset potential shifted in two circumstances as observed by PFE: 1. when the enzyme processed substrates other than PCE and TCE such as DBE and DCP and 2. when the enzyme is inhibited by KCN. Crystallographic studies have shown that the DCP and DBE occupy the same position as TCE in the active site and the shift to more

negative values would indicate that the stabilization of Co in the +2/+3 state occurs over the +1 state.

One hurdle in carrying out quantitative experiments with the existing electrochemical set-up was the escape of the substrate into the headspace which could not be avoided. It was essential to design experiments with mandatory steps of reverting back to a predetermined set of conditions to get a 'base reading' in chronoamperometry experiments by buffer exchange such that 'film loss' could be calculated. This methodology for 'film loss' developed in this chapter is new and utilizes buffer exchanges and injection methods to calculate and correct for liquid substrates; correction for film loss have been carried out for gaseous substrates previously.

Chapter 5: PFE Studies of CODH

5.0 Abstract

Anaerobic Ni-carbon monoxide dehydrogenases (Ni-CODHs) are able to catalyze CO_2/CO interconversion with extremely high turnover rates and are thus of considerable interest to the scientific community to understand their '*design principles*'. For use in technological applications, it is imperative to understand their susceptibility to O_2 which causes irreversible damage. Protein film electrochemistry (PFE) has proved to be useful to understand both substrate and inhibitor interactions with Ni-CODHs and was thus utilized further to investigate the effect of O_2 on them. To make it a general study on the enzyme's susceptibility to oxidants, the interactions of another oxidant, H_2O_2 with the enzyme were also investigated in a similar manner. It was found that the damage was potential dependent with application of higher potentials (>-50 mV) affecting less damage than lower potentials (-520 mV to <-50 mV). It was also found that an enzyme already bound with cyanide was protected partially from damage from O_2 and H_2O_2 .

A nitrogen-containing molecule, nitrous oxide, N_2O , has previously been reported as a substrate with PFE investigations; assignable to its isoelectronic similarity to CO_2 . Interestingly, another nitrogen-containing molecule hydroxylamine, (NH_2OH) shares a structural similarity to H_2O_2 , an inhibitor, but is reported as a substrate. PFE proved to be a powerful tool in assigning the role of NH_2OH in its interaction with Ni-CODH. It was found that whether NH_2OH is a substrate or inhibitor depending upon the potential applied to the system.

Additionally, PFE utilization allowed the maintenance of low potential, otherwise inaccessible with conventional chemical means, which suggested Ni-hydride in the catalytic cycle *via* formate formation during CODH-catalyzed CO_2 reduction.

5.1 Introduction

Anaerobic Ni-containing carbon monoxide dehydrogenase (Ni-CODH) catalyzes carbon dioxide (CO₂)/ carbon monoxide (CO) interconversion as shown previously in Equation 4:¹⁰¹



A value of turnover rate as high as 40000 s⁻¹ for CO oxidation at pH 8 and 70°C has been reported for this class of CODH enzymes^{99, 108} which compares favourably against that reported for aerobic Mo-Cu CODH (~100 s⁻¹).^{106, 109} Carbon dioxide is a difficult molecule to activate, however, it occurs quite efficiently in nature with Ni-CODHs.⁹⁷ Therefore, understanding how Ni-CODHs achieves such is important for designing the best biomimetics for CO₂ reduction.¹⁰² Although much research is dedicated to conversion of CO₂ to fuels, conversion to CO is also a favourable pursuit as CO is an important ingredient of syngas which may be utilized in Fisher-Tropsch processes. The interconversion processes brought about by Ni-CODH is, thus, highly important in the industrial scenario though the irreversible damages by O₂/air, reported from various microorganisms,^{112,158,159} requires investigation.

Previous studies with PFE focused on substrate and inhibitor interactions of anaerobic CODH from *Carboxydotherrmus hydrogenoformans* as functions of applied potentials. As natural substrates, CO and CO₂, were investigated as a function of potential but so was N₂O because of its isoelectronic nature to CO₂. The results proved the active site of CODH capable of working with N₂O as substrate. The nature of inhibition by cyanide (CN⁻), cyanate (CNO⁻) and carbon monoxide (CO) and sulphide (S²⁻) were all found to be potential dependent in previous studies with PFE.^{103a} Given the pertinent nature of investigating the susceptibility of Ni-CODH to O₂ and PFE's previous success with

studying inhibitors, it may be conceivable to utilize PFE to pursue the study of potential dependency of O_2 inhibition. Another oxidant, hydrogen peroxide, H_2O_2 , may also be investigated to make it a general study on oxidants. This chapter details the results obtained from utilizing PFE to study the potential dependency of O_2 and H_2O_2 inhibition on Ni-CODH. Interestingly, it is widely reported that a nitrogen-containing small molecule, hydroxylamine (NH_2OH), is an inhibitor of hydrogen peroxidases because of the similarity of its structure with H_2O_2 (shown in Figure 60). Thus NH_2OH could prove to be an efficient inhibitor however, it has been reported as a substrate of WT and mutant variants in CODH II_{Ch} and CODH $_{Rr}$ by site-directed mutagenesis.^{152, 160} Thus this chapter also outlines the results obtained by investigating hydroxylamine with PFE to clarify its role.

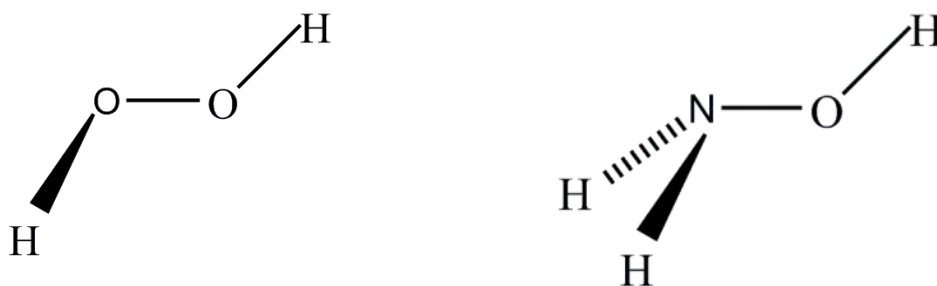


Figure 60: Chem Draw representations highlighting the similarity in structure of H_2O_2 and hydroxylamine (NH_2OH).

The catalytic cycle of Ni-CODH is thought to occur *via* the formation of either a Ni(0) state or Ni-hydride (Section 1.7.4). Insertion of CO_2 into a M-H bond to produce formate has been reported for molecular metal catalysts and for formate dehydrogenases which contains Mo in its active site.^{161,162} It may be envisioned that formate formation by CODH on PGE electrodes will consolidate the role of Ni-hydride in CODH catalysis. The use of PFE with PGE electrodes allows maintenance of low potential

otherwise inaccessible with chemical titrants and should allow experimental conditions conducive to formation of formate to be accessed.

5.2 Results and discussion

5.2.1 Cyclic voltammetry with CODH I_{Ch} and CODH II_{Ch} with CO and CO₂

A substrate which is reduced by the enzyme immobilized on a Pyrolytic Graphite Edge (PGE) electrode shows a reductive current and a substrate which is oxidized by the active site shows an oxidative current in PFE experiments. The profiles for CODH I_{Ch} and CODH II_{Ch} shows the oxidation profile with its substrate, CO, but CODH II_{Ch} does not show any reductive profile with CO₂ due to CO product inhibition being higher in CODH II_{Ch} (Figure 61).^{103a}

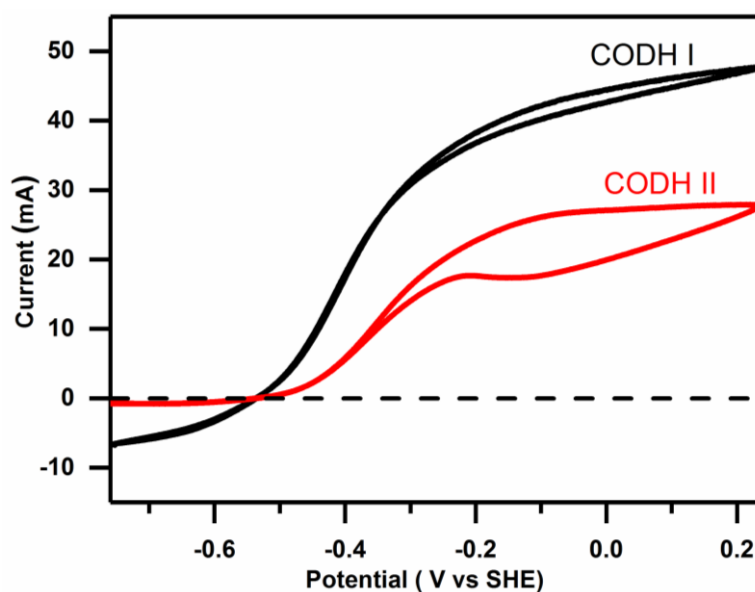


Figure 61: (A) Electrochemical profiles CODH I_{Ch} and II differ because of CO inhibition. CODH I_{Ch} and II profiles were obtained with 50% CO+50% CO₂ in the headspace of the electrochemical cell and it is seen that CODH I_{Ch} shows both an oxidation current and a reduction current (black trace). The enzyme is able to process both CO and CO₂ as substrates. The profile of CODH II_{Ch} (red trace) shows only the oxidation current and the inability to process CO₂ has been assigned to a greater degree of product inhibition from CO. Experimental conditions: MES buffer, pH = 7, T = 25°C and rotation rate= 2500 rpm.

5.2.2 Investigations into O₂ and H₂O₂ as inhibitors of CODH I_{Ch} and CODH II_{Ch}

Chronoamperometry at +140mV and –260 mV vs SHE were undertaken to investigate the potential dependency of inhibition by oxidants, O₂ and H₂O₂. A potential of +140mV ensures the dominance of C_{ox} state in the CODH while –260 mV results in C_{red1} dominance (Figure 14). After an initial recording of data for film loss at the chosen potentials (higher in the +140 mV potential due to the concurrent formation of C_{ox}), the potential was stepped down to –760mV to reductively restore any C_{ox} state which may have been formed (Figure 62). The current was then restored to the initial higher values when the potential was subsequently switched back to +140mV/ –260mV. The damage by O₂ was then assessed by injection of 0.5ml of a O₂-saturated buffer solution which was removed by a flow of CO for approximately ~1000 sec. A short exposure to –760mV and a subsequent potential step to +140mV showed a partial recovery of the current but negligible recovery in a step up to –260mV. This demonstrated that more negative potentials were conducive to irreversible inactivation brought about by O₂ but also suggested that at high potential (+140mV), C_{ox} state may be formed as a means of affording protection to the enzyme. This suggestion is brought about by the fact that the recovery in current (*i.e.* catalytic activity) is seen after an initial step down to –760mV.^{103c} While the results are shown for CODH II_{Ch}, it was verified by similarly conducted experiments with CODH I_{Ch} that the degree of damage brought about by O₂ was the same in both enzymes. The observations are in strong contrast with the observation made with hydrogenases in which oxidizing conditions (more positive potentials) render O₂ most potent.^{34a,163}

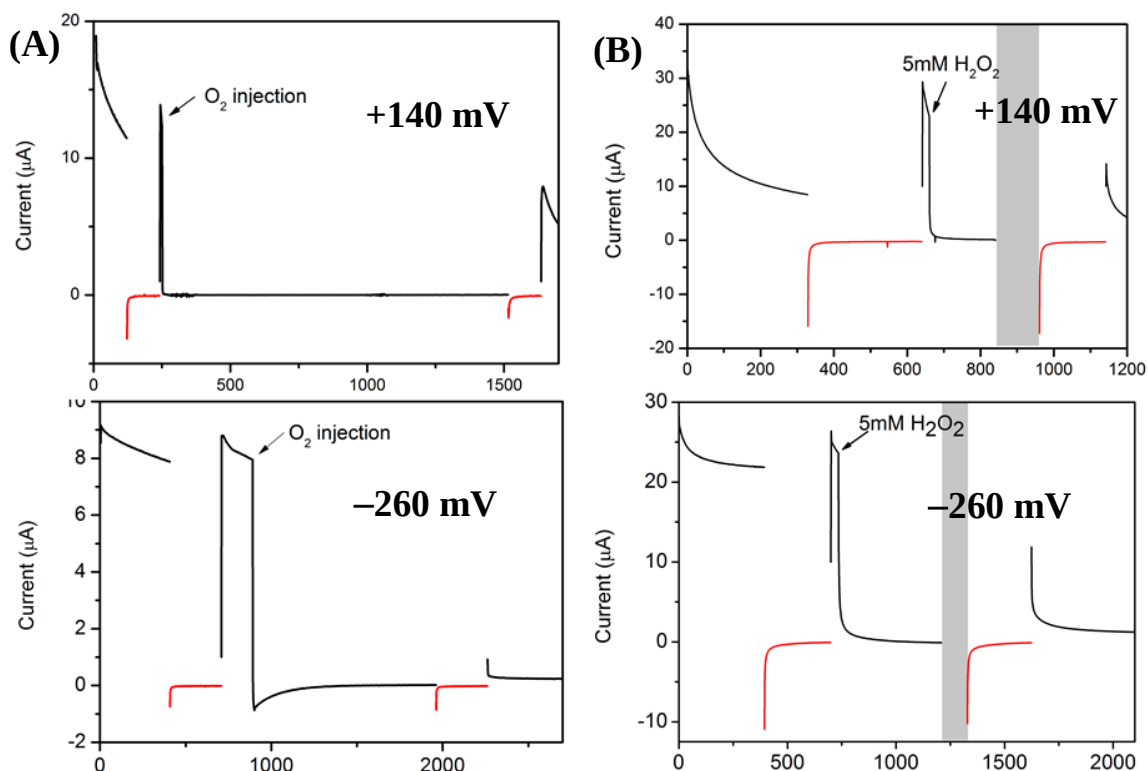


Figure 62: Chronoamperometric profiles of CODH II_{Ch} on exposure to oxidants. (A) Interaction with O_2 at potentials -260mV and $+140\text{mV}$ is reproduced from a study by Wang *et al* by chronoamperometry (with permission).^{103b} The initial black trace in both the upper and lower panel shows the extent of film loss (at 100% CO) that occurs with the loss being higher in the $+140\text{mV}$ potential due to the concurrent formation of C_{ox} . A short exposure to -760mV reductively restores the C_{ox} state to the C_{red} capable of carrying out CO oxidation which is seen by the high current value when the potential is subsequently switched to $+140\text{mV}$ and -260mV . Injection of 0.5ml of a O_2 -saturated buffer solution resulted in an immediate drop in current as the enzyme is damaged by the O_2 . After a $\sim 1000\text{ sec}$ flow of CO and an assumption that O_2 had been removed and a short exposure to -760mV shows a partial recovery in current at subsequent potential step to $+140\text{mV}$ (upper panel) but negligible recovery in a step to -260mV (lower panel). (B) Similarly conducted experiments are carried out with H_2O_2 to expound the interaction that CODH I_{Ch} with the enzyme. Buffer exchanges, indicated by grey regions, were carried out to remove H_2O_2 from the system after damage was observed. Experimental conditions: 100% CO, MES buffer, $\text{pH} = 7$, $T = 25^\circ\text{C}$ and rotation rate = 2500 rpm .

Given, peroxide has been reported as an intermediate component formed during O_2 attack by [Ni–Fe] hydrogenases,²⁸ the generalization of response to this component was investigated with a similar set of experiments as detailed by Wang *et al*^{103b}. At low potentials, O_2 is reduced directly at the electrode producing superoxide which is converted rapidly to hydrogen peroxide. Resolving the possible reactions with superoxide is problematic but PFE experiments to determine how H_2O_2 reacts with Ni-CODH may easily be carried out. The results of H_2O_2 damage variation with potential with CODH II_{Ch} were investigated and are shown in Figure 62B. Buffer exchanges, indicated by grey regions, were carried out to remove H_2O_2 from the system to allow comparison to the experimental results in Figure 62A where O_2 was flushed out by CO flow. Similar results with H_2O_2 (purged entirely of O_2 by purging with in-house N_2 until immediately prior to use) demonstrate that CODH behave with oxidants *via* same mechanism; however, the possibility of O_2 damage to enzymes being due to its conversion to H_2O_2 within the enzyme structure, before reaching the active site, cannot be discounted.

Wang *et al* studied the inhibition brought about in the presence of KCN and reported the potential dependency of the inhibition.^{103a} It was bound when the applied potential ensured that CODH was in a C_{red1} -dominated state and unbound when CODH existed in the C_{red2} -dominated state. Crystal structures of CN-bound CODH show that KCN binds to the C-cluster.^{111b, 28}

In the experimental results shown in Figure 63, film loss is monitored at -260mV. After stabilization was ensured, an aliquot of KCN was added to make the final concentration

of it in the cell 10mM. We may infer that the CN^- is bound to the C-cluster and would offer ‘protection’ against attack by oxidants if the C-cluster is the binding site for them.

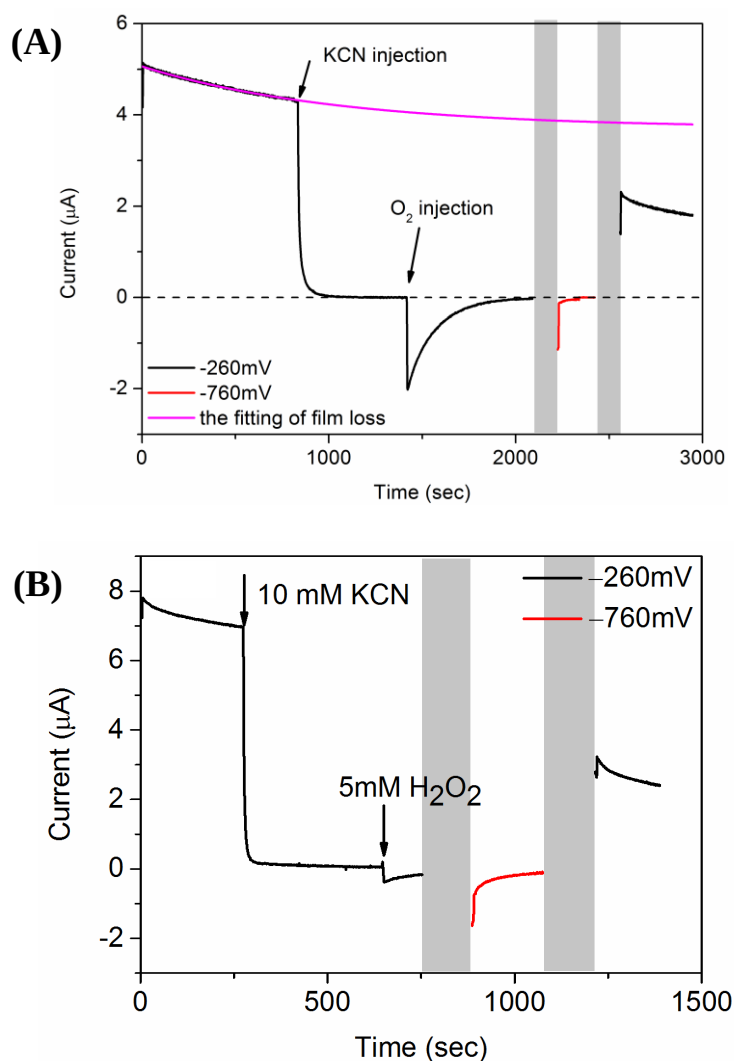


Figure 63: Cyanide protection of CODH II_{Ch} from O₂ and H₂O₂ attack. The black lines indicate the potential was poised at – 260 mV while the red lines are at – 760 mV. An aliquot of KCN solution (giving a final concentration of 10mM in the cell) was injected at -260 mV in both (A) and (B) after assessing degree of film loss. The fall in current was instantaneous in both cases. 5mM of O₂ was added in (A) while the same concentration of H₂O₂ was added in (B). Panel A is reproduced (with permission) from a study by Wang *et al.*^{103b} were then added. The grey windows refer instances of buffer exchanges which was required to remove H₂O₂. O₂ was removed by CO flow in the headspace. The pink line in (A) is extrapolated to indicate the fall in current expected from film loss. A short exposures to –760mV show partial recoveries in currents in both cases. Experimental conditions: 100% CO, MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

Oxygen was introduced in Figure 63A in the form of O₂-saturated buffer solution while H₂O₂ was injected as is from a stock solution to give 5mM final conc. in the cell (Figure 63B). A brief exposure to −760mV was carried out to ‘unbind’ the cyanide. Buffer exchanges ensured complete removal of the cyanide before returning the potential to −260mV and it was observed that the catalytic activity restored to ~50% of the expected value (pink trace is the extrapolated line to track impact of film loss). A conclusion may be drawn: oxidants bind to the C-cluster of the enzyme – a plausible scenario given that both CO and O₂ have π -acceptor properties: although oxidants do bring about damage to other parts of the enzyme as evidence by ~50% recovery.

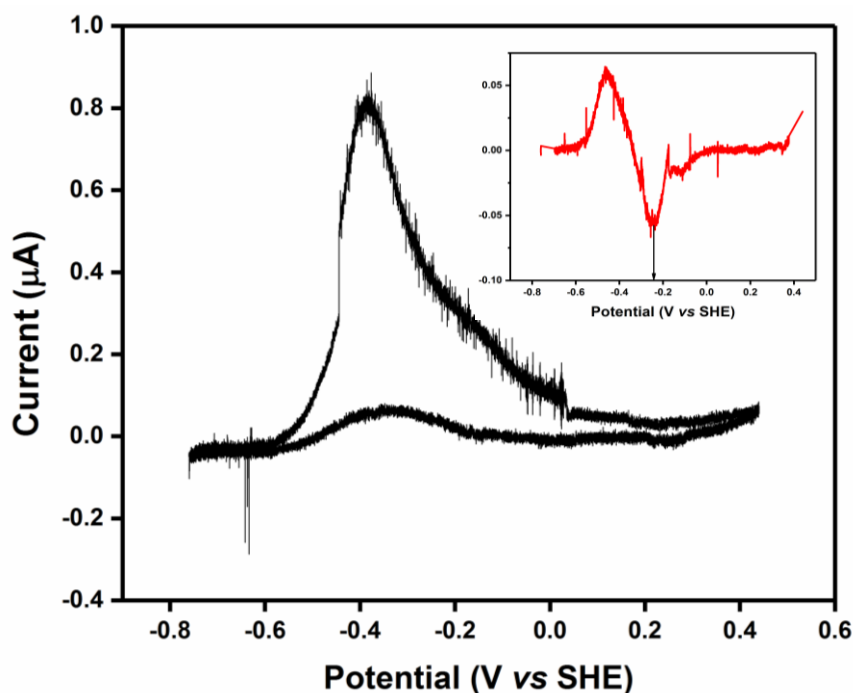


Figure 64: Damage to CODH to long-term exposure to low levels of O₂ (<25ppm). At potentials higher than −0.4 V vs SHE, the enzyme is unable to sustain activity and it has been observed on many occasions. This observation has been made with both CODH I_{Ch} and CODH II_{Ch}. The inset shows the derivative of the reverse scan done at 1mV s^{−1}. The recovery from the damaged system occurs at −0.242 V. Experimental conditions: 100% CO, MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

A study by Leger *et al*¹⁵⁸ reiterated that anaerobic CODH from different microorganisms are susceptible to O₂ but the concentration which renders them irreversibly damaged also varied from sample to sample. Although proper investigation was not carried out, it should be noted that long-term exposure to low levels of O₂ (<25ppm) does render the profile of CODH from *Carboxydotherrmus hydrogenoformans* different obtained by that which has not be exposed. Figure 64 shows the damage to CODH I_{Ch} from long term exposure to O₂ (<25ppm). At potentials higher than – 0.4 V vs SHE, the ‘damaged’ enzyme is unable to sustain activity as observed on many occasions. This observation has been made with both CODH I_{Ch} and CODH II_{Ch}. Interestingly, it has been possible to recover the profile of the un-damaged CODH from the damaged CODH by repeated exposure (5/10 minutes each) to low potential (–760mV vs SHE). An E_{switch} value of –0.242V was obtained for CODH as was obtained in Chapter 3 for hydrogenases and it is more negative than values obtained earlier in the group by members investigating anaerobic inactivation in which C_{red1} converts to C_{ox} by 1-electron oxidation. From the results given in Figure 62A and Figure 64, the damage by O₂ seems to occur by reacting with the enzyme in such as manner as to stabilize the C_{ox} state: an pathway which is available upon exposure to low levels of O₂ and high-potential (+140mV). At –260mV this pathway does not seem to be available and the damage is irreversible.

5.2.3 Cyclic voltammetry with CODH I_{Ch} and CODH II_{Ch} with hydroxylamine as inhibitor and substrate

As an inhibitor: The CODH I_{Ch} and CODH II_{Ch} profiles are reproducible but with hydroxylamine (NH₂OH) delivered into electrolytic solution by injections shows that the profile is notably changed as potential becomes more positive than 0.06 mV vs SHE

(the exact potential is dependent on the scan rate). At high potentials, the fall in activity of the enzyme occurs and is noted by drop in current. This is shown in Figure 65 and is attributed with the inability of the CODH to process CO – an inhibition brought about the presence of NH_2OH in the system. Traces with either of the CODH are similar but not the same and highlights the importance of potentials on the systems. The raw data is shown as normalized data to allow comparison: two points of differences are easily identified: 1. the inhibition of CODH I_{Ch} occurs at $\sim 50\text{mV}$ more positive values and 2. the recovery occurs at $\sim 100\text{mV}$ more positive values. The binding of NH_2OH is reversible and is evidenced when the potential sweep is taken to the most negative value to generate C_{red2} . When the potential sweep to the more positive end is reinitiated, the same profile is seen again. Thus, NH_2OH cannot bind to the C_{red2} state and the profile of CO-electrocatalytic profile is obtained until high potential values ($> 0\text{ V vs SHE}$) are reached where the inhibited-profile begins. With CODH II $_{Ch}$, a further experiment was conducted (not shown) with 50% CO+50% CO_2 to detect whether the recovery of the inhibited state of the enzyme from the inhibited state extends to region of CO_2 reduction, *i.e.*, when enzyme is in C_{red2} state. However, no such observation was made. Thus, the presence of NH_2OH affects the C_{ox} - C_{red1} equilibrium but does not affect C_{red1} - C_{red2} equilibrium. In either of the enzymes, inhibition and recovery occurs at potentials near the C_{ox} - C_{red1} equilibrium potential and thus do not support the hypothesis that NH_2OH inhibition is comparable to that by H_2O_2 as inhibitor despite structural similarities between the two.

As a substrate: Since hydroxylamine is reported as a substrate with conversion to ammonia,¹⁰⁴ it was decided to investigate the reduction reaction through PFE. It was chosen to investigate the detection of ammonia. The technique for detection of ammonia

is detailed in Section 8.6.3 (Ion-selective electrode), and since the detection limit is 10ppm, 12 hour-run with 1-2 μ l of CODH I_{Ch} (13.3 mg/ml) on electrode modified by carbon nanotubes¹²⁰ was considered sufficient. CODH I_{Ch} was chosen over CODH II_{Ch} as CO –product inhibition is lower in CODH I_{Ch} .

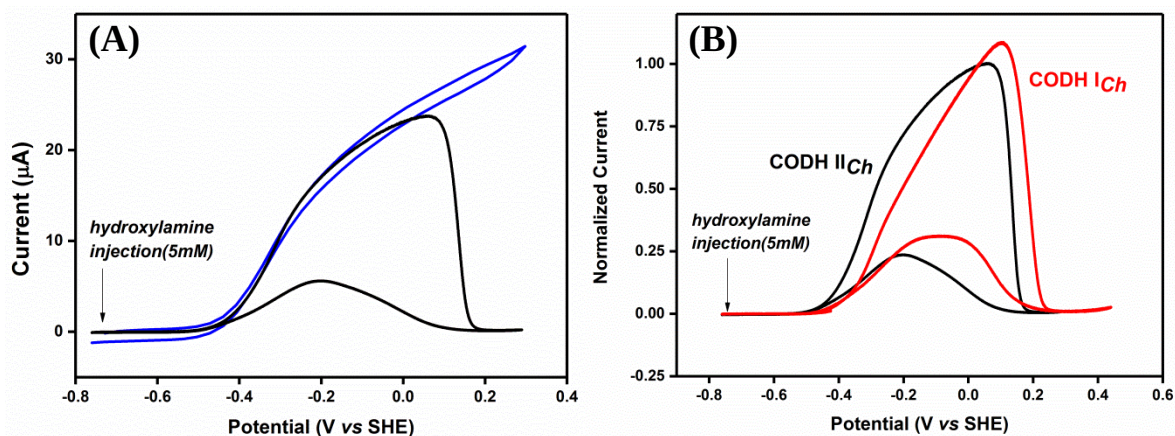


Figure 65: Hydroxylamine inhibition of CODH I_{Ch} and CODH II_{Ch} . Panel A shows the electrochemical profile of CODH II_{Ch} (blue trace) to which hydroxylamine was injected to give a 5mM concentration at the most negative end of the potential sweep during the ‘dead time’ of the instrument. The black trace shows the scan that followed-it may be noted that the profile is to the preceding blue trace except at high potentials at which the current drops. The reverse sweep shows recovery in the activity to give a trace immediately after. Panel B compares hydroxylamine inhibition of CO activity between CODH I_{Ch} and CODH II_{Ch} . The data is shown as normalized data to allow comparison: two points of differences are easily identified-1. the inhibition of CODH I_{Ch} occurs at $\sim$ 50mV more positive values and 2. the recovery occurs at $\sim$ 100mV more positive values. Experimental conditions: 100% CO, MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

A low potential of -1V vs SCE was maintained to provide maximum driving force conducive for reduction to occur. A control experiment in which there was no enzyme on the electrode was also carried out. The samples were analysed and it was found that :0.0000071 moles of NH_3 were produced by this method while half the quantity (0.00000355 moles of NH_3) were detected in the control. To investigate the source of NH_3 in the control experiment, MES buffer was also submitted for sampling in which

no NH_3 was detected but the addition of hydroxylamine (NH_2OH) gave a positive reading negating the observation that it was possible to detect NH_3 and quantify by this method. Another method of was needed to verify NH_2OH as a substrate.

The results of the alternate method is shown in Figure 66. A chronoamperometry was carried out at -1 vs SCE to provide maximum driving force and 100% CO_2 was maintained in the headspace. A reductive current is seen to grow in ~ 550 s which may be attributed to the time needed for the buffer to equilibrate with CO_2 . The gas flow was then stopped and the current reduced gradually to zero with Argon to flush away CO_2 . First an injection of 1 ml of buffer was carried out to act as control after which 1ml of hydroxylamine solution was injected to give a cell concentration of 10mM. It was anticipated that if CODH were able to process hydroxylamine as substrate a reduction current would be seen. However, since a reduction current was not seen, 100% CO_2 was again introduced to investigate whether the enzyme was still active. A reduction current was seen upon the introduction of CO_2 again demonstrating that the enzyme was still viable. This result is not conclusive as well and NH_2OH may be a substrate of CODH whose rate of reaction is too low to be detected by PFE. The ability of CODH to process NH_2OH has been attributed to the similarity of the structure of CODH at the active site to that of NH_2OH reductases,¹⁰⁴ and though the product was not unequivocally detected as being formed by PFE (*via* ion-selective electrode) or the reductive current seen by chronoamperometry in this study, there is reason to believe that the similarity in structure at the active site could account NH_2OH as a substrate.

5.2.4 Investigations of CODH I_{Ch} and CODH II_{Ch} Inactivation with hydroxylamine

Inactivation at high potential of CO-oxidizing CODH I_{Ch} was seen with hydroxylamine. The inhibition was first investigated with regards to NH_2OH concentration. A series of chronoamperometry experiments was conducted at -0 V vs SHE with NH_2OH injections carried out to give required concentration once a steady film was obtained with 100% CO (Figure 67A). It was seen that the rate of inactivation as well as the extent of inhibition dependent on the concentration of NH_2OH which was injected into the system. The rate of inhibition is seen to be proportional to the 1st power of $[NH_2OH]$ and indicates role of NH_2OH in the rate-determining step of CO-inhibition brought about by NH_2OH .

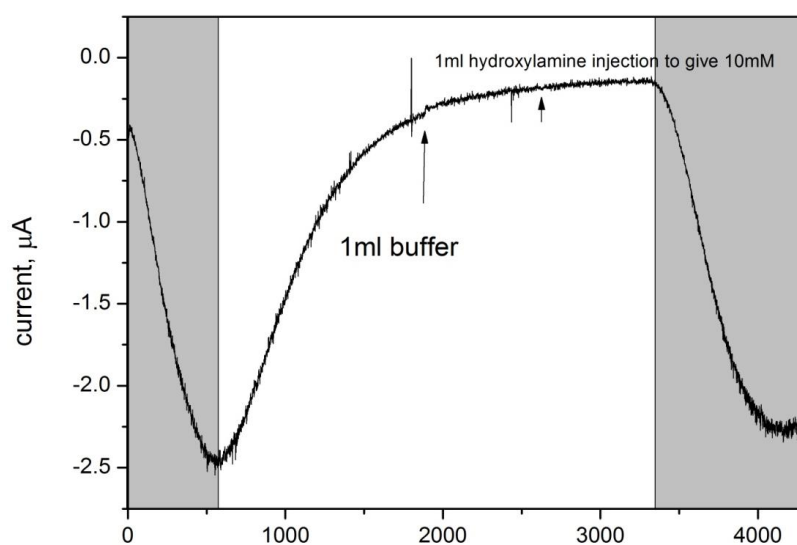


Figure 66: Chronoamperometry to test hydroxylamine as a substrate of CODH I_{Ch} . Chronoamperometry was carried out -1 vs SCE with grey regions indicating 100% CO_2 in the system. Reductive currents were seen with CO_2 in the system which slowly reduced when Argon was used to flow the CO_2 away. Once the current reached $0.5 \mu A$ (white region), 1 ml of buffer solution was injected to act as control after which 1 ml of hydroxylamine was injected. No reduction current was seen, although introduction of CO_2 resulted in the recovery of the cathodic current demonstrating that the enzyme was still viable in the set-up. Experimental conditions: 100% CO_2 , MES buffer, pH = 7, $T = 25^\circ C$ and rotation rate = 2500 rpm.

A further experiment was carried out to test the importance of CO concentration in the inhibition of CODH-catalyzed CO oxidation. A set of cyclic voltammograms are seen in Figure 68 were obtained at increasing concentrations of CO maintained in the headspace (10%, 30%, 70% and 100% CO). The graph was normalized with respect to the highest current immediately prior to inhibition effect of NH_2OH .

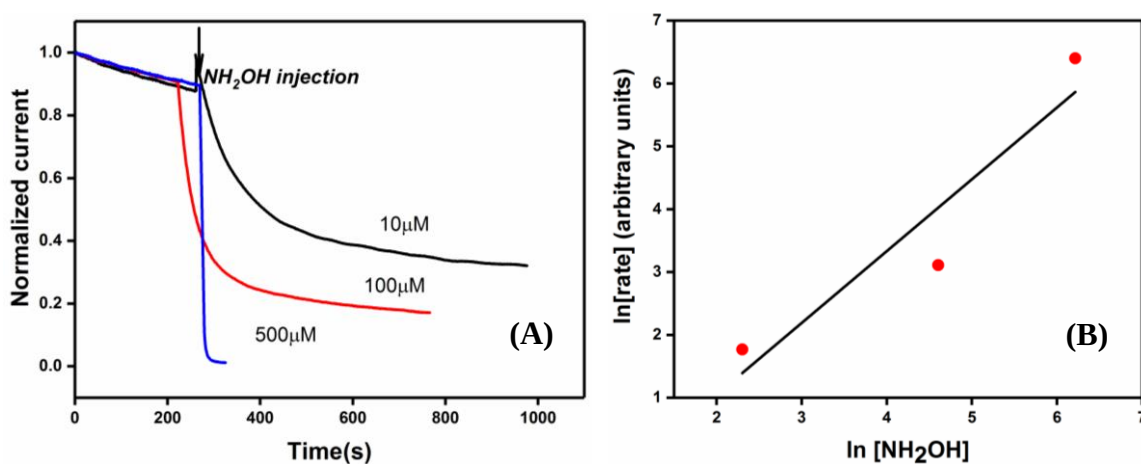


Figure 67: (A) Effect of variation of NH_2OH concentration on the rate of inhibition. The extent of inhibition upon CODH-catalyzed oxidation of CO also increased with the current to which the system equilibrates in the presence of NH_2OH and its concentration. (B) A higher concentration increased the rate of inhibition. Experimental conditions: 100% CO, MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

Since PFE does not give absolute rate, the relative rates were utilized to plot against $\ln [\text{CO}]$ to give a straight line. It is seen that the order reaction of the reaction is '−1'. The inhibition is thus of complex form. It may be due to the fact that the enzyme is driven to be in the C_{red} state in the presence of CO: an increase in concentration of CO would increase the drive. The relative rates were obtained from the slope of the first few seconds (3-6 s) of the obtained inactivation profiles.

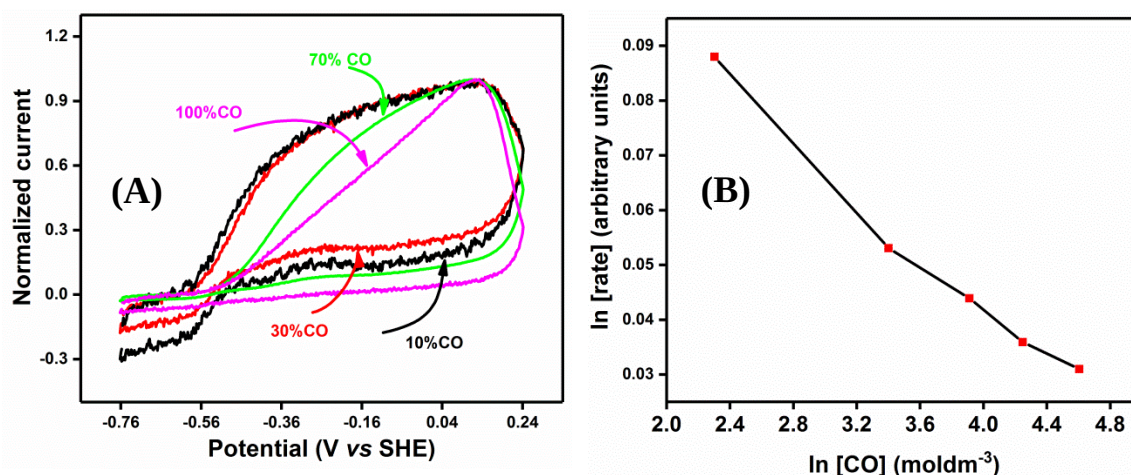


Figure 68: The effect of CO concentration on inhibition by NH_2OH of CO oxidation. Panel A shows the rate of inactivation at various concentrations of CO are obtained from the first few seconds of cyclic voltammograms which had been normalized to allow comparison. Panel B shows the plot of the ln of rate vs ln [CO] which show that the reaction is of 1st order kinetics. The extent of inhibition upon CODH-catalyzed oxidation of CO also increased with the current to which the system equilibrates in the presence of CO and its concentration. Experimental conditions: MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

Figure 69 shows the results investigation in to the potential dependency of the inhibition brought about by hydroxylamine. The red traces are rates of ‘film loss’ at -260 mV before inactivation at increasingly more positive values of applied potential shown by the green traces. The rates are inferred from the fitting of the green portions to the exponential decay by software Origin 8.0. Prior to each set of -260 mV and potentials for inactivation (-150 mV to 0 V), a short exposure of -760mV vs SHE were carried out to recover any C_{ox} which may have been formed in the previous set of potential poises. It is seen that the rates of inhibition increases with increase in potential– a significant inference is that electron transfer is involved in the rate-determining process.

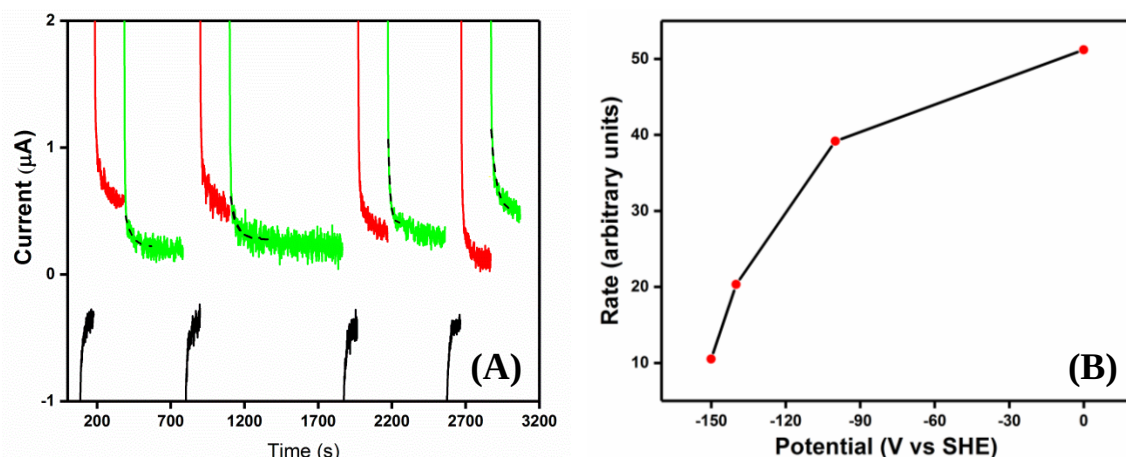


Figure 69: Evaluation of potential dependency of the rate of inhibition. The red traces are currents generated as a result of potential poised at -260 mV for CODH I_{Ch-} catalyzed CO oxidation. The traces were used to correct for film loss and yielding the green traces which are inactivation at increasingly more positive values of applied potential. The rates are inferred from the fitting of the green portions to the exponential decay fitting option in Software Origin 8.0. The black cathodic traces are short potential poises of -760 mV vs SHE to recover any C_{ox} which may have been formed in the previous set of potential poises. Experimental conditions: 100% CO, MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

5.2.5 Investigations of recovery of CODH activity with hydroxylamine

Chronoamperometry experiments with hydroxylamine injections were carried out as shown in Figure 70A, one with the injection made shortly after poisoning at the high potential and one after the complete formation of C_{ox} . Two different electrocatalytic profiles for E_{switch} (Figure 70B and Figure 70C) are obtained. The recovery profiles in the former case showed two profiles of corresponding to two E_{switch} values—one at ~ 0 V corresponding to C_{red1} formation from C_{ox} and the other at which must correspond to one of recovery of NH_2OH -dissociated state from the NH_2OH -bound state. Thus, introduction of NH_2OH brings about formation of C_{ox} and a small amount of NH_2OH -bound state. In the latter case, only one recovery profile *i.e.* only one value of E_{switch} is obtained upon injection of NH_2OH after complete conversion of the enzyme to the C_{ox} state. This result is significant—hydroxylamine does not get a chance to bind when C_{ox}

is completely formed and must, thus, be binding to a state prior to formation of C_{ox} but which is formed only at high potential.

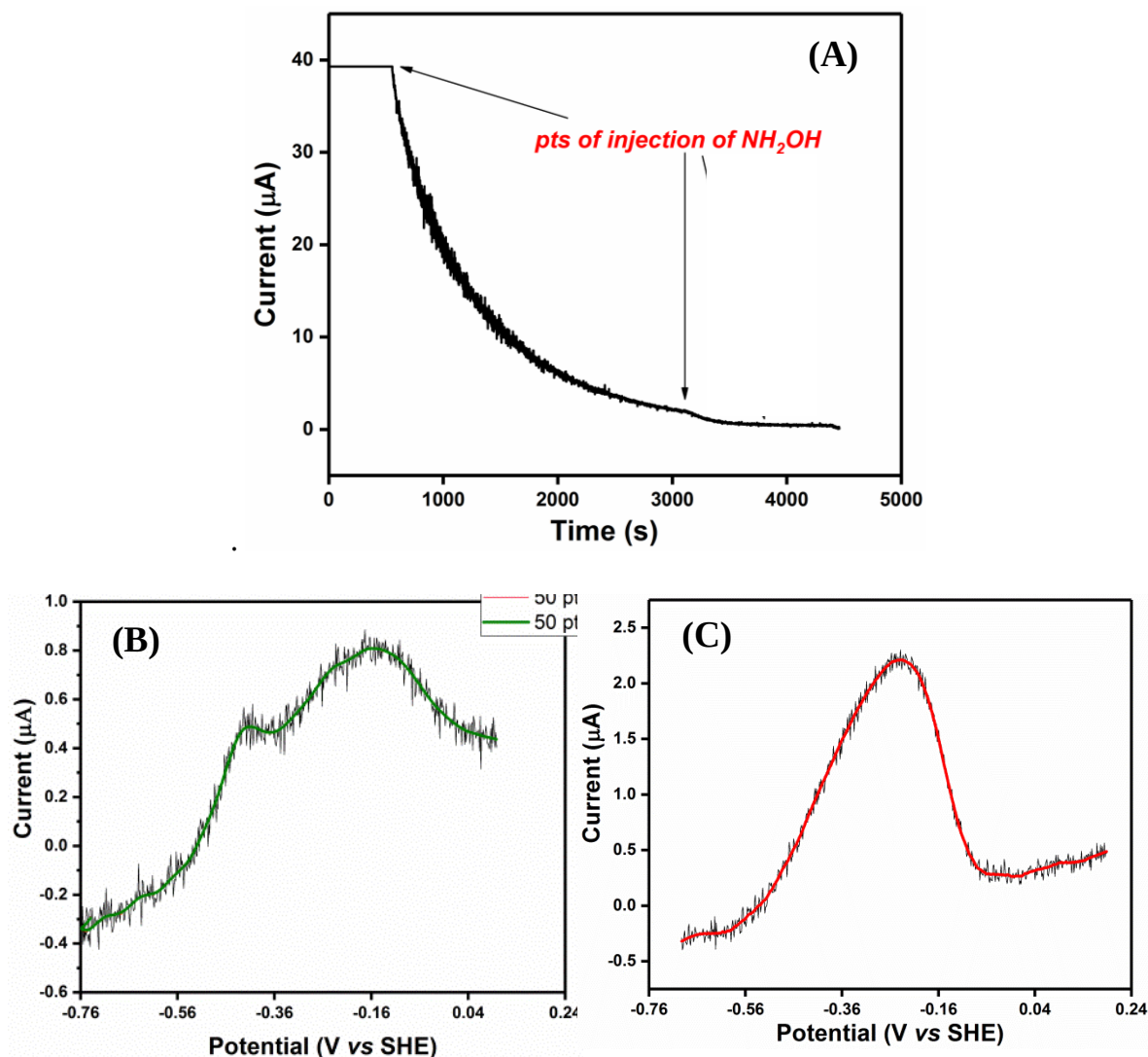


Figure 70: (A) Hydroxylamine injection may be carried out in two instances. It may be carried out soon after a stable film is ensured or it may be after the high potential is maintained until C_{ox} is formed completely. (B) Electrochemical profile after hydroxylamine injection is carried out immediately after the potential pause at +140 mV. Two humps are seen. (c) Electrochemical profile after hydroxylamine injection is carried out after complete formation of the C_{ox} state. One hump is seen. Experimental conditions: 100% CO, MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

Figure 70 shows that the recovery at different pH follows a similar trend between C_{ox} – C_{red1} conversion as in CODH I_{Ch} and CODH I_{Ch} – hydroxylamine system, though the CODH I_{Ch} – hydroxylamine system manifests at a much more negative value. The acceleration of C_{ox} formation and its stabilization brought about by NH_2OH (E_{switch} values are shifted to more negative values) may be a result of that NH_2OH forming an adduct from which recovery to C_{red1} is more ‘difficult’ than from C_{ox} itself. Also, there is a 59mV shift with in the value of E_{switch} indicating that H^+ is involved along with e^- in the inactivation/reactivation processes.

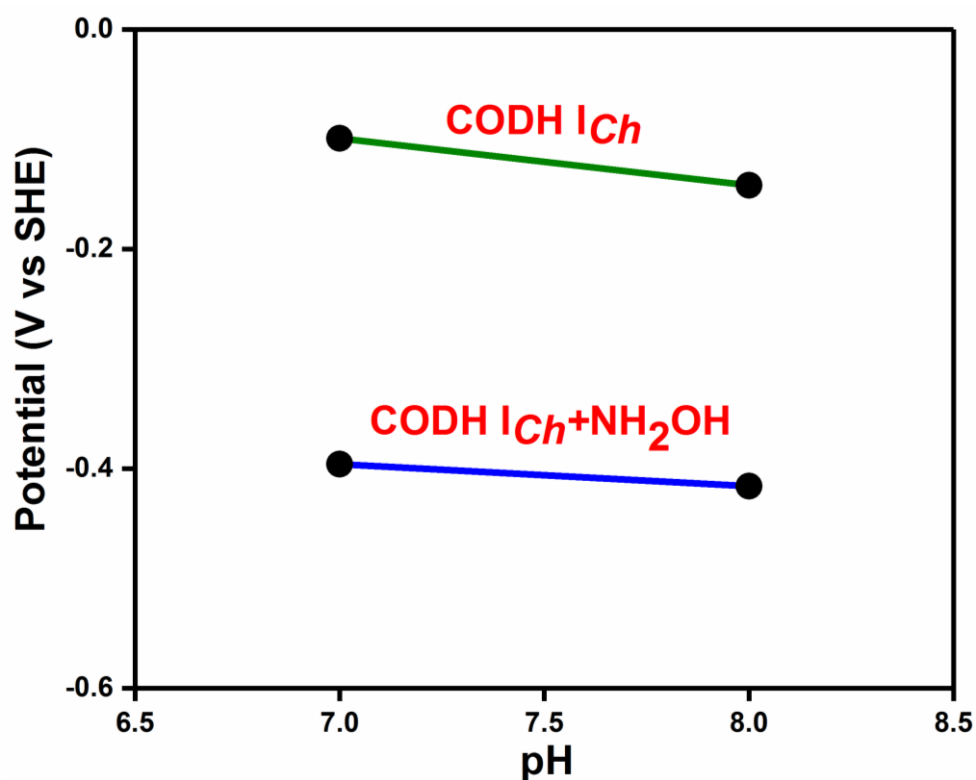


Figure 71: E_{switch} values at different pH. The given trace is obtained by plotting E_{switch} values of CODH I_{Ch} vs pH (with 100%CO flow in the headspace). A chronoamperometry is carried out with potential poised at high value (+140 mV). Once the current falls to 0 V (either after complete formation of C_{ox} or inhibition by NH_2OH), a reverse sweep from the high potential to a lower potential (–760 mV) yields the electrocatalytic profile from which the E_{switch} values are derived. Experimental conditions: MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

5.2.6 Role of H₂ in the catalytic cycle

Formate production: It was proposed that formate formation by Ni-CODH would provide reasonable evidence for involvement of a Ni(II)-hydrido species, because insertion of CO₂ into M–H bonds to produce formate is well established for molecular metal catalysts.¹⁶⁴ Figure 71A shows the ¹H NMR spectrum of the solution resulting from the reduction of CO₂ by CODH I_{Ch} adsorbed on a MWCT-modified PGE electrode held at –760 mV over a period of 3 days. A characteristic ¹H peak for HCOO[–] was observed at 8.4 ppm similar to that obtained for a pure solution of formate at the same pH (upper panel). Neither was any formate detected in experiments in which the MWCT-modified PGE electrode, with or without CODH I_{Ch} adsorbed, was held at open circuit potential under CO for 4 days. The middle panel in Figure 71 shows the results of experiments conducted as in the upper panel but with a flow of N₂ instead of CO. Furthermore, adding a small aliquot of pure potassium formate solution to the NMR tube resulted in a single peak at 8.4 ppm, thus establishing unambiguously that the product is formate (lower panel).

Hydrogen (H₂) as an inhibitor: Wang *et al*^{103b} determined that H₂ evolving during CO oxidation brought about by CODH I_{Ch} at rates of 0.16 s^{–1}. Since study H₂ is formed by similarity with water-gas shift (WGS) reaction it was thought pertinent to investigate the inhibitory actions, if any, of H₂. A chronoamperometry was carried out at –1 vs SCE and the H₂ in headspace was incrementally increased from 10 to 50% while maintaining 50% CO₂ in the headspace (Figure 71B). No impact was observed upon the reductive current, indicating that H₂ is not an inhibitor of the CODH-catalysed CO₂ reduction reaction. If it were, the rate must be extremely low in order for there to be *no impact* on the reductive current.

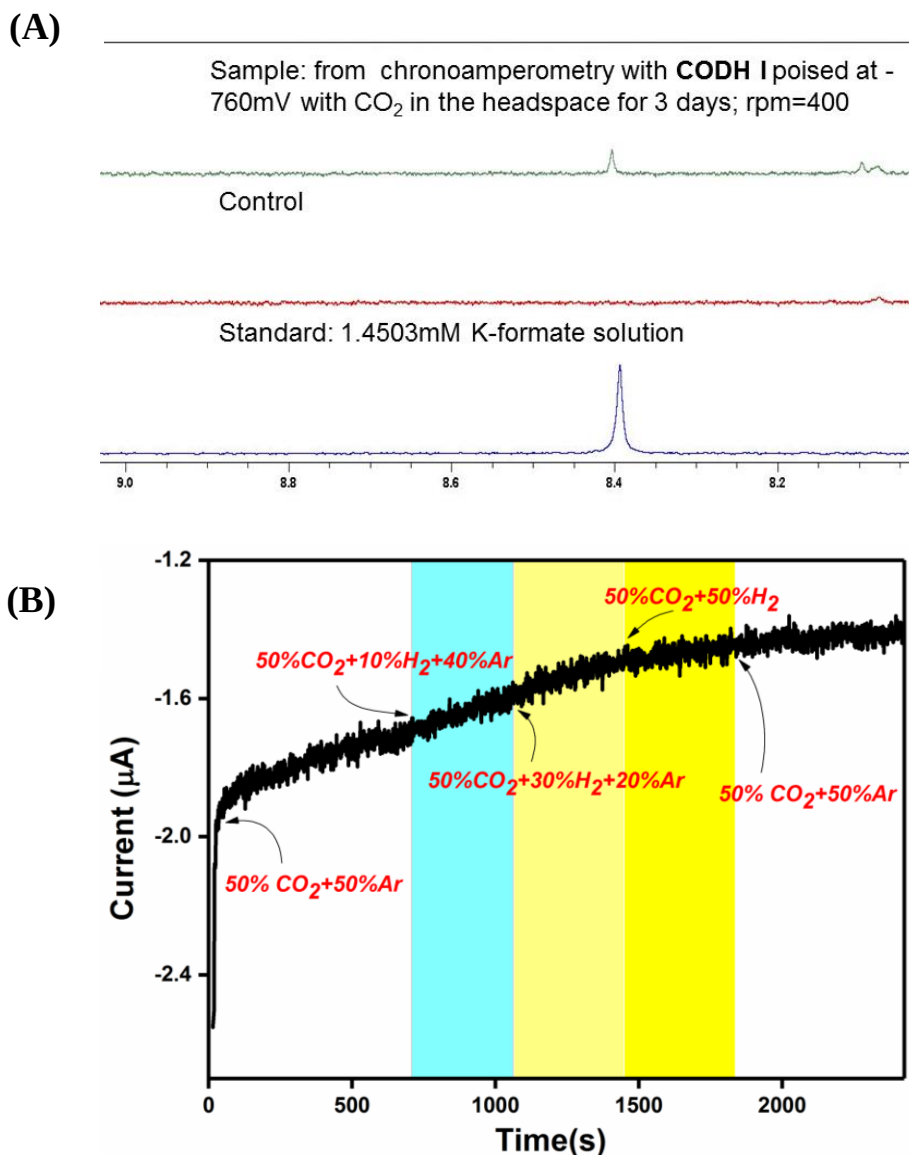


Figure 72:(A) Proof of formation of formate by CODH I_{Ch} adsorbed on a MWCT-modified PGE electrode at -760 mV. The ¹H NMR spectra of the solutions sampled from electrolytic solution obtained under different conditions are shown. The upper panel shows the solution collected from the cell with a three-day flow of CO₂ in the headspace. The PGE electrode was modified with MWCT upon which the enzyme had been allowed to adsorb as described previously.¹²⁰ Experimental conditions: 25 °C, 0.2 M MES (pH 7.0 and 45.5% D₂O and 55.5% H₂O), electrode rotation rate 400 rpm. The middle panel shows the spectrum obtained for the control experiment conducted under identical conditions as that in upper panel with N₂. The lower panel shows the spectrum of 1 mM potassium formate in 100% D₂O under the same conditions. Peak at 8.4 ppm demonstrate the formation of formate during catalysis. (B) H₂ is tested by chronoamperometry as an inhibitor to CO₂ reduction catalyzed by CODH I_{Ch}. Chronoamperometry in which H₂ in heaspace incrementally increased from 10 to 50% shows no effect on the cathodic current obtained by 50% CO₂ in the headspace. Experimental conditions: MES buffer, pH = 7, T = 25°C and rotation rate = 2500 rpm.

5.3 Conclusion and Perspectives

The activation of CO₂ to bring about formation to fuels/CO is difficult to achieve and much interest exists to learn from Nature in which carbon monoxide dehydrogenases (CODHs) bring about CO₂/CO interconversion efficiently. Anaerobic Ni-containing CODHs operate with high turnover frequencies though much needs to be learned about their O₂-susceptibility before they may be used in technical applications.

Unlike cyanide and cyanate which are not redox active at the PGE electrode, O₂ cannot be studied as an inhibitor at low potentials without it also being reduced at the electrode. Hence, PFE experiments by Wang *et al*^{103b} focused on high potential (+140 mV) and a relatively lower potential (-260 mV) to study O₂ inhibition. This study focuses on similarly designed experiments on H₂O₂ inhibition which served to prove that Ni-CODH reacts similarly to these oxidants. Conclusion may be drawn that the C_{redI} is susceptible to irreversible inactivation. Most significant from the experiments was that the high potential seemed to afford protection to the enzyme from the damage caused by the oxidants. Addition of O₂ or H₂O₂ at this potential (even at low concentrations) most probably favours the formation of inactive form of the enzyme which can be reactivated upon reduction; this may be the hypothetical state or the defined redox state, C'_{ox} (shown in Figure 14).^{103b} Whatever the state, the cyanide-protection experiment proves C-cluster to be (one of) the targets of O₂/H₂O₂ damage.

Inoue in 2013 reported CODHs from both *Carboxydotherrmus hydrogenoformans* and *Rhodospirillum rubrum* as being hydroxylamine reductases.¹⁰⁴ The ability to do so has been attributed to the structural similarity of the active site with hybrid cluster protein (from *E. coli*) which has confirmed hydroxylamine reduction activity. Contrary to these

reports, PFE revealed inhibitory action of hydroxylamine when CODH I_{Ch} and II were subjected to high potential irrespective of whether it was injected at low or high potential. They differed in hydroxylamine added product being less stable in CODH I_{Ch} than in CODH II_{Ch} (inhibition occurred at $\sim 50\text{mV}$ more positive values and the recovery occurs at $\sim 100\text{mV}$ more positive values). To appreciate is the fact that whereas the $C_{ox}-C_{red1}$ equilibrium is visibly changed, the $C_{red1}-C_{red2}$ equilibrium is unaffected. Moreover, the inhibition brought about by NH_2OH seems to be potential dependent indicating that electron transfer is involved in the mechanism. Chronoamperometry experiments with NH_2OH revealed that higher concentrations brought about higher rates of inhibition with 1st order kinetics while the rates were inversely proportional to the concentration of CO. The inverse relation is interesting and maybe due to the tendency for the C_{red1} state to dominate in the presence of CO even at high potential.

Electrocatalytic profiles for recovery from inhibition yields interesting results: they support the idea that NH_2OH inhibition brings about acceleration of C_{ox} formation which is supported by the observation made above that electron transfer is involved in the inhibition mechanism. To be noted is the fact that NH_2OH binding must be occurring to a state which is accessible at high potential but is not C_{ox} . The formation of an adduct between NH_2OH and CODH is thought to occur at this high potential. Similar to the O_2 experiments, binding may occur at a hypothetical state C'_{ox} ^{103b} or to a completely new hypothetical state C''_{ox} which maybe proposed here(as mentioned in Figure 14).

Hydroxylamine is known to bind with ketones to form oximes and bring about the conversion of Fe^{3+} to Fe^{2+} . Based on these, the state to which it binds must be a

CO₂-bound CODH in which there is a Fe³⁺. The iron in the 3+ state must be the displaced Fe in Figure 12 (Section 1.7.2) and accessible at the high potential. The hypothetical state C''_{ox} is then indicated to be the CO₂-bound state as CO is oxidized and in which the high potential has brought about the conversion of Fe²⁺ of the active site to Fe³⁺. The two hypothetical states need verification and may also be referring to the same state: further results may be obtained by EPR or IR which may follow unpaired electrons in the former case or NO and CO bond stretching in the latter case.

Chronoamperometry experiments could not verify NH₂OH as a substrate and detection of ammonia with an ion-selective electrode was possible but not conclusive. The active site of CODH is similar to hydroxylamine reductase and thus it cannot be discounted as a substrate. The *non*-detection by PFE may then be due to extremely low rate of conversion of hydroxylamine to ammonia. More conclusive are the inhibition experiments and may be due to structural similarity to hydrogen peroxide (H₂O₂). NH₂OH acts as a competitive inhibitor of peroxidases by combining with the Fe of the active site. Given the observation that sulfide inhibits rapidly when the potential is more positive than -50 mV^{103a}, it is interesting that sulfide, oxygen and nitrogen (all of which are highly electronegative) brings about reversible inhibition in a similar manner. This may be important in providing '*design principles*' for CODH-inspired biomimetics.

Lastly, the ability of CODH I_{Ch} to generate formate from CO₂ is difficult to verify by chemical means due to the low reduction potential ($E < -0.5$ V at pH 7.0). This problem is overcome by PFE which showed that a small amount of formate was indeed formed. This provides reasonable evidence for involvement of a Ni(II)-hydrido species, because insertion of CO₂ into M-H bonds to produce formate is well established for molecular

metal catalysts.^{42,43} Given this new similarity to water-gas shift reaction, it was investigated whether H₂ is an inhibitor of CODH-catalysed CO₂ reaction by PFE. It was found that H₂ had no inhibitory actions or if any, did so at extremely low rates undetectable by PFE.

Chapter 6: *CrHydA1* and *Cpl*

ImpedanceResults

6.0 Abstract

This chapter outlines a new and interesting approach to investigating enzymes which are immobilized on electrodes. Electrochemical Impedance Spectroscopy (EIS) provided means to obtaining valuable information that was not accessible using standard DC voltammetric techniques. More specifically, the use of this frequency dependent technique allowed the determination of the enzyme coverage and theoretical turnover frequency at the zero-current potential *during* catalysis by immobilized enzymes on electrodes. Use of this technique on *CpI* hydrogenase, a [Fe–Fe] hydrogenase with [Fe–S] clusters as part of the active site and an electron relay system, and on *CrHydA1*, a simple [Fe–Fe] hydrogenase with only one [Fe–S] cluster as part of the active site but not the electron relay system, allowed the evaluation of the role of the electron relay system. The results are presented in the form of time-dependent and time-independent resistances which vary among the two enzymes when a range of potential is applied. The conclusion is drawn that the active site of both enzymes should work at the same turnover rate, but it is the interaction with the electrode that limits the observed turnover frequencies for *CrHydA1*. Given the experimental conditions, it was found *CpI* was inherently two-three times more efficient than *CrHydA1*. This work could be easily extended to other enzymatic or catalytic systems immobilized on electrodes with similar electrochemical set-up to that utilized in Protein Film Electrochemistry (PFE) experiments.

6.1 Introduction

To understand the ‘*design principles*’ of an electron-transporting enzyme, it is conceivable to measure the response to a bias, analogous to what is done in the case of evaluation of electronic circuitries. In these evaluations, information is obtained by Electrochemical Impedance Spectroscopy (EIS) where impedance is a generalized resistance term and is the opposite of conductance – the electronic term expressing the ease of electron flow. We have used EIS to investigate hydrogenases which catalyse the oxidation and production of H_2 with activities that may rival platinum^{28, 53-54} and are inspirational in the quest for future electrocatalysts, especially in artificial photosynthesis processes. Electrochemical impedance spectroscopy uses a small amplitude sinusoidal AC voltage to measure the impedance components at given values of the electrode potential^{122, 165}. The electrochemical set-up is a three-electrode set-up connected to an analyser which measures the time lag, $\emptyset$, experienced between the current and applied voltage and Z_0 , the amplitude of impedance displayed by the system. Impedance, Z , is defined by the formula $Z = Z_0 \exp[j\emptyset]$ from which the real and imaginary components of the impedance may then be obtained: the real portion is denoted Z' and the imaginary portion by Z'' and a plot of Z' vs Z'' results in a point on a Nyquist plot. Another feature of this technique is the ability to alter the frequency at which these measurements are taken – a range of frequencies from 10^{-3} to 10^6 Hz may be employed, each point may be plotted on the same Nyquist plot to give a trace whose characteristic shape enables the analysis of the cell. Analysis is accomplished by comparison to an virtual assembly of resistors and capacitors and inductors which give the same trace. The assembly is known as the equivalent circuit. Care must be taken that the assembly reflects the actual biophysical processes of the system under study so that the analysis reflects the processes within the system.

Enzymes chosen for this study are known to be reversible electrocatalysts on Pyrolytic Graphite Edge (PGE) electrodes in Protein Film Electrochemistry (PFE) experiments with minimum overpotentials required to switch the current direction on either side of the equilibrium potential (also known as the zero-current potential). It would be significant to know how rapidly the catalytic cycle can turn back and forth at the equilibrium potential and EIS allows this measurement by means of analysing response to application of small amplitude sinusoidal AC voltage, just as is done in case electronic circuitries evaluations.

Terms for expressing efficiency at zero-current potential are established for systems other than enzymes *e.g.* exchange current density obtained by extrapolating the exponential current – overpotential relationship in a Tafel plot for irreversible electrocatalysts and the Marcus electron self-exchange rate which is obtained at zero driving force for reactions involving chemically identical species differentiated only by their oxidation states. For enzymes evaluated by PF, which measures and records net electron flow, it is not possible to learn the rates of electron transfer involved at equilibrium potential from the value of ‘zero’ obtained at that potential. Were it possible to measure the rates, efficiencies of different enzymes can be compared as is done by evaluating exchange current density and electron self-exchange rate values. This comparison can also serve as the basis for selecting enzymes to be inspirations for production of biomimetic catalysts. We envisioned the term *electrocatalytic exchange constant* for this value and, in reality, it should encompass a combination of long-range electron transfer (ET) efficiency and inherent catalytic proficiency of the active sites of enzymes. As this value should reflect ET efficiency within the enzyme, the

presence/absence of Fe–S electron relay systems within the enzyme should be significant.

Two enzymes, *CrHydA1* and *CpI*, were chosen for this study. They belong to a class of enzymes known as [Fe–Fe] hydrogenases and differ by presence/absence of Fe–S electron relay systems within them. They both contain a unique bimetallic active site known as the H–cluster which contains six Fe atoms in two domains: a 2Fe domain that is the site for H₂ activation and a [4Fe–4S] cluster domain that presumably acts as an internal electron buffer within the H–cluster as shown in Figure 73.⁷⁰ The figure shows *CpI* which additionally contains an electron-transfer relay consisting of three [4Fe–4S] clusters and one [2Fe–2S] cluster.¹¹ The relay allows long-range electron transfer (ET) between the H–cluster and the protein surface (and a ferredoxin redox partner^{73b}), and it may be significant that it branches into two pathways to make external contact at two different parts of the protein surface.

In contrast, *CrHydA1* contains only the H–cluster: in the living cell, it receives electrons directly from Photosystem I *via* a small [2Fe–2S] ferredoxin known as PetF⁷⁴ with no accessory structures within to support ET. In PFE experiments, the surface of the PGE electrode would serve as the redox partners to both the enzymes and assuming similar and fast ET from the PGE to the enzyme, cyclic voltammograms (CVs) would be reflective of the activities occurring *via* the active site. This project aims to understand and differentiate between the roles of the relay and the active site by distinguishing between the time-dependent chemical steps occurring at the active site and the ‘wire-like’ conductivity (time-independent property) of the relay.

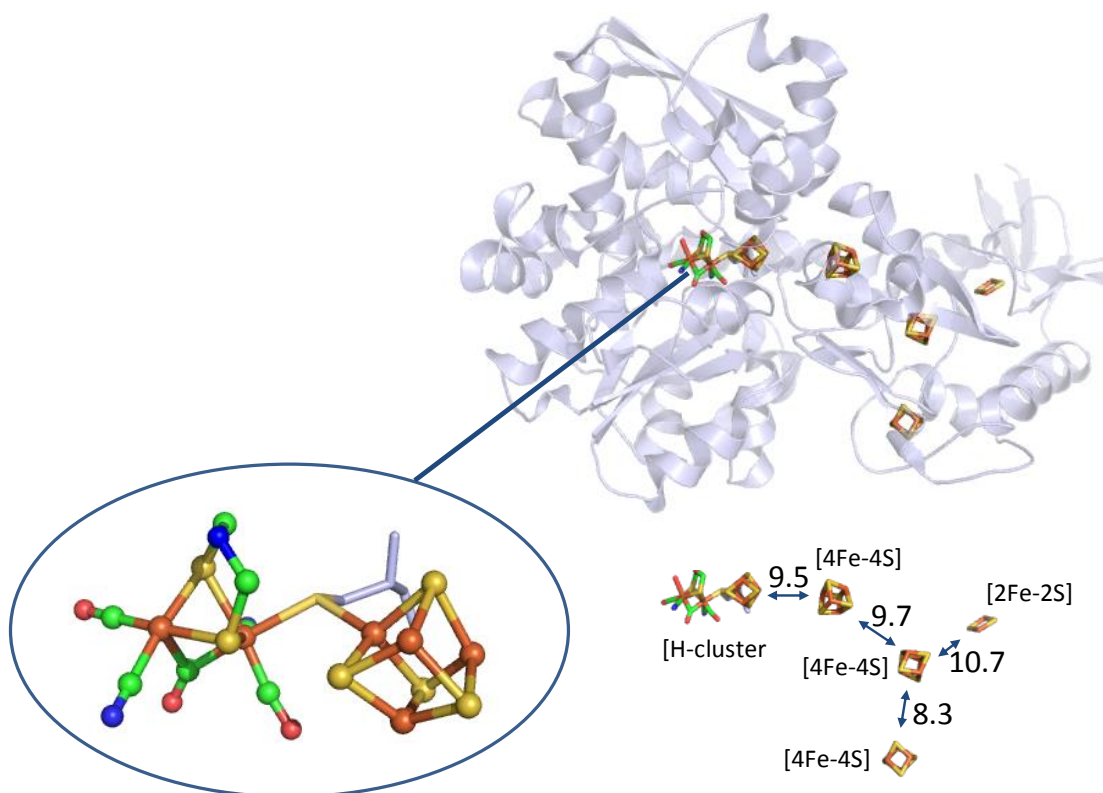


Figure 73: Structure of the [Fe–Fe] hydrogenase I (*CpI*) from *Clostridium pasteurianum* (PDB code: 3C8Y)¹¹. A close-up view of the active site H–cluster is also shown (lower left) along with the [Fe–S] cluster relay and inter-site distances (lower right) measured in Ångström. This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶

6.2 Results of PFE: CV of *CpI* and *CrHydA1* on PGE

All experiments were carried out at 0 °C, pH 7.0, under strictly anaerobic conditions using a rotating disc PGE electrode (in 0.10 M phosphate buffer as electrolytic solution) at which *CpI* and *CrHydA1* adsorb tightly. The air-tight three-electrode electrochemical cell was subjected to flows of 100% H₂ in the headspace to provide catalytic activity close to optimal and electrode rotation at 1000 rpm ensured that H₂ mass transport was not limiting.

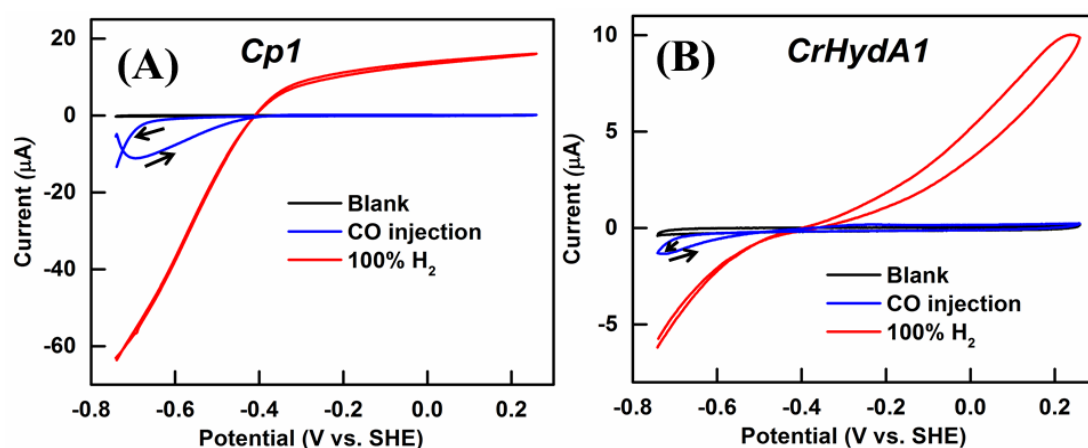


Figure 74: Cyclic voltammetric profiles of [Fe–Fe] hydrogenases: (A) *CpI* and (B) *CrHydA1*. The red traces are obtained on PGE electrodes upon equilibration with 100% H_2 in the headspace. They indicate both oxidation and reduction is carried out by the enzymes depending upon the potential applied, albeit with different potential-dependency of the response. When CO is injected (blue trace), both the enzymes are unable to carry out oxidation and the reduction profiles begin at a more negative potential. This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶ Experimental conditions: pH = 7 (0.10 M phosphate buffer), T = 0 °C, rotation rate = 1000 rpm, scan rate = 20 mVs⁻¹.

The catalytic electrochemistry of *CpI* and *CrHydA1* is highly reproducible to that shown as red traces in Figure 74 (A) and (B). In both cases, the steady-state catalytic current changes direction sharply on either side of the equilibrium $2H^+/H_2$ potential. However, notable differences are evident between *CpI* and *CrHydA1* activities: *CpI* appears particularly efficient at catalysing H_2 evolution whereas the H_2 oxidation current levels off within 0.2 V of the reversible value. Although not cutting the x-axis as sharply as *CpI*, *CrHydA1* catalyses both H_2 production and H_2 oxidation reactions at similar rates for a given overpotential. Due to complications that may arise from anaerobic inactivation at high potentials,¹⁶³ it was decided to restrict subsequent EIS measurements and evaluation thereof to potentials below – 0.250 V vs SCE to avoid complications.

Both hydrogenases are inhibited by carbon monoxide (CO), which binds tightly at a potential more positive than -0.5 V, but is released at a potential < -0.6 V. The blue traces are CO-inhibited profiles of the enzymes taken a few scans later with the same electrolytic solutions and films of the relevant enzymes that gave the red traces. Before the start of the blue trace in which the potential is swept from the most negative to the most positive value and then back to the negative value, the flow of H_2 was stopped and 5ml of a CO-saturated solution was injected into the electrolytic solution (already saturated with H_2 from the previous scans) to give an initial CO concentration of $77\mu\text{M}$. The flow of H_2 had to be stopped to make sure that CO was not taken away by its flow during EIS measurements. As the potential of the CO-saturated electrolytic solution is swept from extreme negative to more positive, we see that the trace cuts the x-axis where the red trace cuts but there is no current observed corresponding to oxidation occurring. The enzyme has been inactivated in the presence of CO– the scan back shows that a much more negative potential $< -0.6\text{V}$ is required to recover any activity at all, proof that CO had been bound to the enzyme when in the more oxidized state obtained by application of potentials > -0.41 V. Application of potentials more negative than -0.6V changes the oxidation state of the enzyme so that CO cannot bind anymore.

Based on this, EIS experiments, requiring CO to act as benign and reversible ‘off/on switch’ to identify the impedance components unique to electrocatalysis, were carried out as follows: Chronoamperometry with potential held constant at -0.250 V vs SCE for 10 min to ensure that the CO had bound before switching to the equilibrium potential at which EIS was measured.

6.3 Results of Electrochemical Impedance Spectroscopy (EIS)

6.3.1 Nyquist Plots at Equilibrium Potential

Figure 75 shows Nyquist plots provided by the system's response upon the application of small perturbation modulation and angular frequencies to the same three electrode set-up used to give the voltammograms in Figure 74. The modulation amplitude was chosen as the best compromise to obtaining conditions closest to the equilibrium potential without creating adverse noise.

Four different experimental conditions (blank PGE electrode, CO-inhibited enzyme on the PGE electrode, *CpI* immobilized PGE and *CrHydA1* immobilized electrode) are plotted on the same graph to compare and contrast between. The expanded plot shown in Figure 75B highlights the high-frequency region in which the resistance provided by the leads to the cell and the solution is given by the (extrapolated) intercept on the left at the Z' axis by the arcs. For all the systems, this intercept value, R_s , is identical and is as-expected because the same cell, electrode and solution were utilized to carry EIS measurements. The measurements were also carried out in the same glovebox. The exactness imparted confidence to look at the other portions of the graph to discern differences assignable to biophysical processes brought about by the enzyme.

The right-hand intercept of arc at the Z' axis gives the combined resistances of the previously obtained R_s and R_{ct} , the resistance provided by the double layer at the electrode. The R_{ct} resistance is frequency-dependent, *i.e.*, its contribution to total impedance value varies according to the frequency at which the measurement is taken and is due to the capacitive nature of the double layer.

The value of R_{ct} is the resistance offered by a fully charged double layer and occurs at lower frequencies than R_s . As the frequency is lowered, the double layer gets sufficient time to charge and discharge as the sinusoidal voltage is applied while ions in solution do not require that time.

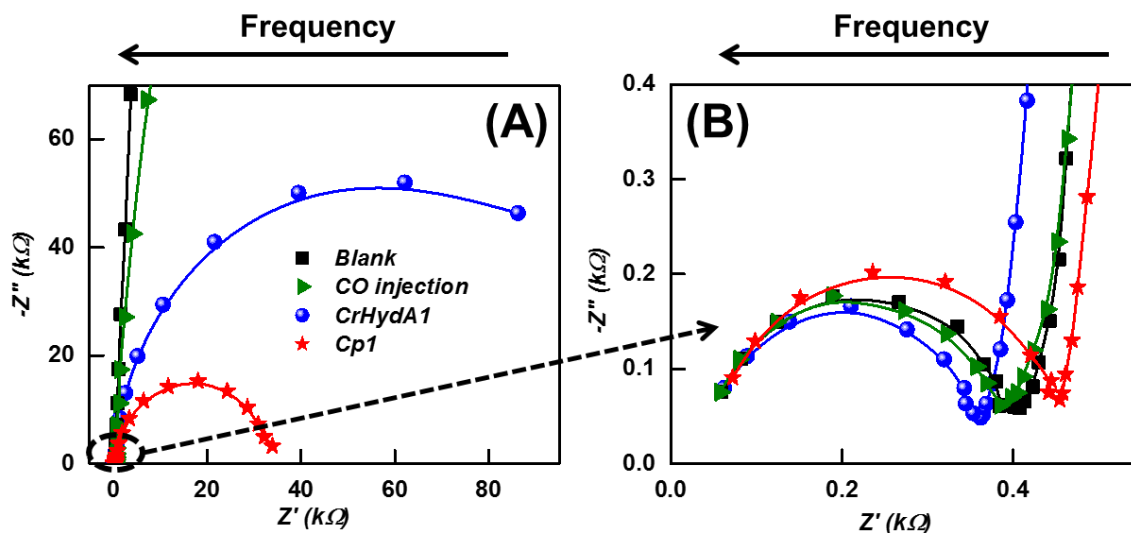


Figure 75: EIS spectra for *CpI* and *CrHydA1* adsorbed at a PGE electrode under 1 bar H_2 . The recordings were undertaken at equilibrium cell potential (zero- current) in the frequency range 0.1 mHz to 100 kHz. At high frequency (see expanded plot 74B), the results obtained for *CpI* and *CrHydA1* are similar and resemble the results obtained for a blank PGE electrode and that observed when CO is introduced to inhibit electrocatalysis (shown in the figure is the *CpI* profile as CO-inhibited *CpI* and *CrHydA1* display the same profile). This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶ Experimental conditions: pH= 7 (0.10 M phosphate buffer), T = 0 °C, rotation rate = 1000 rpm, scan rate = 20 mVs⁻¹.

The slightly differing values of R_{ct} among the four different experimental conditions may be supposed to arise from the differences in the surface characteristics that arise at the electrode under the various conditions. The slight nature of the variances in the R_{ct} values gives us assurance to interpret the remaining data as relating to specific electrocatalytic activities.

To note is the fact that the similarity shown by the high- frequency arcs in the Nyquist plot (Figure 75B) deviate in behaviour at much lower frequencies to give almost straight lines in the case of blank and CO inhibited enzymes, and semicircular arcs of different radii for the two enzymes catalysing $2\text{H}^+/\text{H}_2$ interconversion. The almost straight lines of the blank electrode and enzymes unable to undertake catalytic activities due to inhibition by CO in the system are diffusion related characteristics which are not the subject of this thesis but can be noted down as being due to semi-infinite diffusion of charged particles (Warburg element)¹³⁴.

The difference in the radii of the semi-circular arcs in the low-frequency region is significant. We can assume that it provides information of the inherent, time-dependent and time-independent components and behaviour of the enzyme under zero overpotential. With no driving force, the rates of the forward and reverse reaction are the same and are reflective of the electron-transfer capabilities of each of the enzyme. We make an assumption here that the active sites of the enzymes behave in the same way due to the similarity in the composition and orientation of the active site in the protein framework of the enzyme (Section 1.5.5)⁷¹ and that the main difference among them arises from the electron transfer process from the surface of the protein to within the enzyme.

To infer the significance of the differences in the semi-circular arc radii of the two enzymes, it is imperative to understand how electrons flow through the enzyme systems are represented by the limits of the arc. At the high-frequency limit, the impedance response is simply R_{ct} and similar for both enzymes with and without CO: with the uninhibited enzyme, the frequency is sufficiently high that the enzyme is unable to

contribute through catalytic turnover as occurs with a blank and a CO-inhibited system. The effect of the presence/absence of the Fe–S clusters leading upto the active site is not immediately evident as the active site is *not* catalytically active. At the low-frequency limit (reached by CpI but not by CrHydA1 which would require much lower angular frequencies than instrumentally feasible), the response reflects the resistance, denoted R_e , to electron flow from the protein surface to an active site which is operating under fully catalytic conditions (care is taken to subtract the values of R_s and R_{ct} during analysis). This frequency-independent resistance quantifies the difficulty of moving electrons from the electrode to the active site and may reflect such factors as distance, medium and the influence of a Fe–S relay^{15b, 17}. Given factors other than the relay are constant, it may be inferred that to be fully active CrHydA1 requires *more time* than does CpI due to the absence of the Fe–S cluster electron relay system in CrHydA1. Since the R_e value of CrHydA1 is higher (the diameter of the arc of CrHydA1 is bigger than that of CpI), the difficulty of moving electrons within CrHydA1 from the electrode to the active site is higher.

The responses at intermediate frequencies along the arcs reflect the increasing and decreasing contributions from the frequency-dependent catalytic activity. The capacitance C_e measures the frequency-dependent impedance and reflects the importance of rate-determining chemical steps ranging from binding/release of H_2 to atom/ion transfer and electron transfers at the active site that are coupled to these steps.^{167 168} Expressed in another way, R_e represents the DC resistance always offered by the combined electron relay system (when it exists) and active site, whereas C_e is analogous to a time– dependent charging and discharging of the active site (a Faradaic process because catalytic cycling enables movement of electrons).

6.3.2 Modelling Catalytic Electron Transport by Equivalent Circuit Analysis

To get precise values of R_s and R_{ct} (only estimations can be obtained from Nyquist plots) and to evaluate the magnitude of R_e and C_e , modelling of an equivalent circuit is required as discussed in Section 2.1.5. Essentially arcs represent resistances (R) and capacitors (C) in parallel¹²². As the first arc is placed at a distance from the origin along the Z' axis, the equivalent circuit contains a resistor, R_s , placed in series to the arrangement of R_{ct} and C_{dl} in parallel. In the experiments involving the blank electrode and CO-inhibited enzymes, the almost straight line indicates the requirement of R_{ct} and C_{dl} to be part of a Warburg circuit as mentioned in Section 2.1.5.2. The term W corresponds to the Warburg element which represents the semi-infinite diffusion of the charged particles.

The second arc in the low-frequency region represents the second arrangement of R and C components in parallel. As this low-frequency arc appears when catalytic activity is *not* inhibited by CO shows, the R and C components are R_e and C_e elements discussed in Section 2.1.5.2. Figure 76 is the compilation of the elements discussed above into a complete equivalent circuit diagram which represents the entire catalytic charge transport process at the electrode-enzyme- solution region.

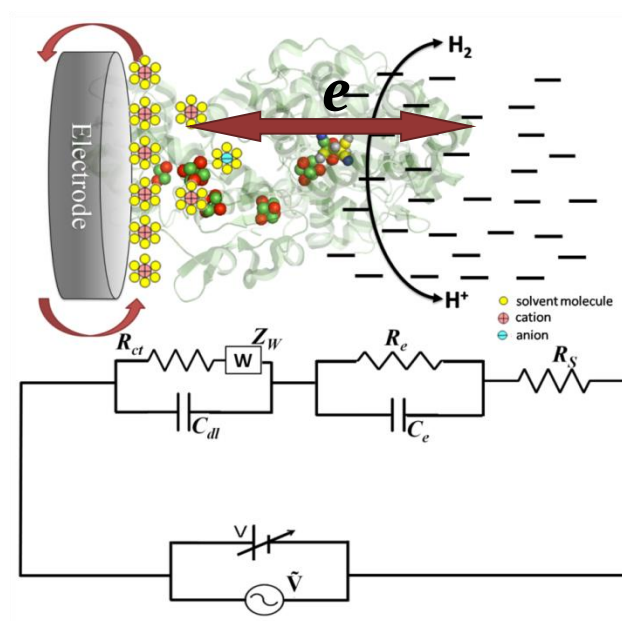


Figure 76: Equivalent circuit used to fit the Nyquist spectra obtained for *CpI* and *CrHydA1* hydrogenases attached to PGE electrode. The scheme highlights the dominance of circuit elements in the different regions of charge transfer. This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶

Although many combinations are possible to yield the two arcs as seen in the Nyquist plot, the need to base the circuit on the biophysical processes that occur in the system makes it prudent to consider the total circuit as three portions in series; the cell resistance, the impedance due to the electrical double layer and the impedance that describes catalytic electron flow through the enzyme, each of which are revealed in turn as the frequency is lowered.

To obtain the values of the components of the whole circuit, the experimental Nyquist spectra for the enzymes on PGE electrode at equilibrium potentials was fitted by MATLAB software. The best fit obtained had error less than 1.3% and the value of the parameters obtained by this fit are given in Table 6.

Table 6: Parameters obtained by fitting the experimental Nyquist spectra for *CpI* and *CrHydA1* on PGE electrode (at equilibrium potentials) are given in the table. The equivalent circuits were designed and the corresponding fit was made by MATLAB software. This table is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶

	$R_s (\Omega)$	$R_{ct} (\Omega)$	$C_{dl} (\mu F)$	$R_e (k\Omega)$	$C_e (\mu F)$
blank	57.5	350.2	0.031	—	—
CO inhibition	57.5	350.1	0.031	—	—
<i>CpI</i> (H₂)	57.5	410.7	0.022	28.6	14.8
<i>CrHydA1</i> (H₂)	57.5	338.3	0.038	119.8	10.8

Most notable from Table 6 is the much lower R_e value for *CpI* compared to that of *CrHydA1* which shows that electron flow through *CpI* is more facile. Although the qualitative inference is the same as *via* inspection of the diameters of the arcs in the Nyquist Spectra, the quantitative value is of significant importance as this R_e value can also then be used to calculate the exchange current density i_0 .¹⁶⁹

$$i_0 = \frac{RT}{nF(R_e)} \quad \text{Equation 30}$$

where R , T , n , and F are the universal gas constant, temperature in Kelvin, overall number of electrons taking part in the reaction and Faraday's constant, respectively. For enzymes, it may be proposed that this i_0 value corresponds to the previously proposed term *electrocatalytic exchange constant* which expresses the efficiency of the enzyme (Section 6.1).

For *CpI* and *CrHydA1*, the i_0 values of $27.7 \mu A \text{ cm}^{-2}$ and a nearly 5-fold lower value of $6.07 \mu A \text{ cm}^{-2}$ were obtained. However, to give context to these values for expressing efficiencies requires knowing the electroactive coverage, Γ , for each case; the

knowledge of which will allow the exchange turnover frequencies, k_{ex} , of the enzymes to be determined from the formula:¹⁶⁹

$$k_{ex} = \frac{nF\Gamma}{i_0} \quad \text{Equation 31}$$

Neither CpI nor CrHydA1 are present at high enough coverage to exhibit non-turnover signals (due to simple, non-catalytic oxidation and reduction of internal electron-transfer components) which would have allowed calculations of electroactive coverage, Γ (Appendix 1). However, an independent value of Γ may be obtained from a Bode plot as discussed in the following section.

6.3.3 Bode Plots at Equilibrium Potential

Figure 77 shows the Bode plots (phase angle plotted against frequency) for CpI and CrHydA1 at the equilibrium potential. The time constant obtained at peaks of the plots are related to the electrocatalytic exchange turnover frequency k_{ex} . For CpI, the peak in the phase angle corresponds to a higher frequency (f) than CrHydA1 signifying a smaller time constant. Quantitatively, using $k_{ex} = 2\pi f$, the k_{ex} values for CpI and CrHydA1 are 157 s^{-1} and 25 s^{-1} , corresponding to 78 and 12 molecules $\text{H}_2 \text{ s}^{-1}$ respectively. These are useful but approximate values erring on the high side, as the modulation amplitude can never be zero (in the experiments carried out, a modulation amplitude 10 mV rms was utilized and equates to $E_{eq} \pm 5 \text{ mV}$).

Furthermore, this k_{ex} value allows, by back-calculation, the value of the electroactive coverage to be known during the experiments conducted. We obtained 0.91×10^{-12} moles cm^{-2} for CpI, and 1.26×10^{-12} moles cm^{-2} for CrHydA1. These low values are consistent with the inability to observe any signals due to Fe–S clusters under non-catalytic conditions. Of note is the fact that while i_0 is a value dependent upon

electrocatalytic coverage, k_{ex} is an inherent property of the enzyme which is related to the ease of electron flow through the circuitry of the enzyme. The i_0 values can now be adjusted to be per mole: a value of $21.98 \mu\text{A}$ is obtained for CpI while a ~ 3 -fold lower value of $6.67 \mu\text{A cm}^{-2}$ were obtained for CrHydA1. As inference may thus be drawn that CpI is slightly more than three time efficient as CrHydA1 in processing of H_2 .

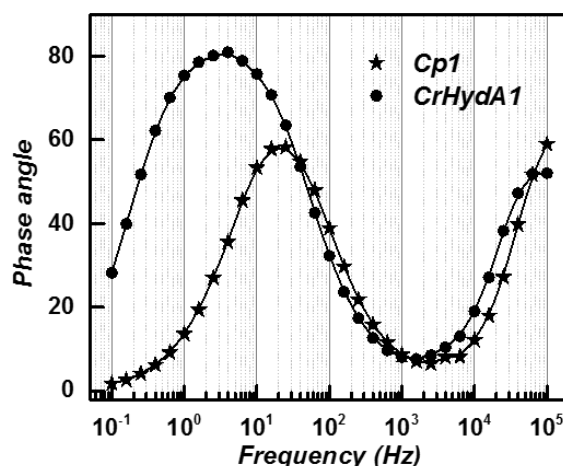


Figure 77: Bode representation for CpI and CrHydA1 at equilibrium potential. The symbols in the plots are experimental data points and the lines are included to guide the eye. This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶ Experimental conditions: pH = 7 (0.10 M phosphate buffer), T = 0 °C, rotation rate = 1000 rpm, scan rate = 20 mVs⁻¹.

6.3.4 Nyquist Plots over a Potential Range

Impedance measurements were carried out at various potentials and the results/Nyquist plots are shown in Figure 78 for both CpI, and CrHydA1. The blue circles represent the systems at their equilibrium potentials.

Applying an increasing overpotential in the proton-reducing direction causes the low frequency semicircles to contract in size and cut the x-axis at increasingly lower Z' values on the right. This is as expected because with increasing driving force, less time would be needed for the enzyme to be fully active. Applying an increasing overpotential in the oxidizing direction, however, displays contrasting behaviour of the two enzymes.

While CrHydA1 displays the expected behaviour in which the increasing driving forces causes the enzyme to become fully active much faster, CpI manifests the opposite behaviour. Figure 79A shows how R_e values vary within a narrow potential range on either side of the equilibrium value. The results were obtained by fitting the Nyquist spectra to the standard model for the circuit shown in Figure 76.

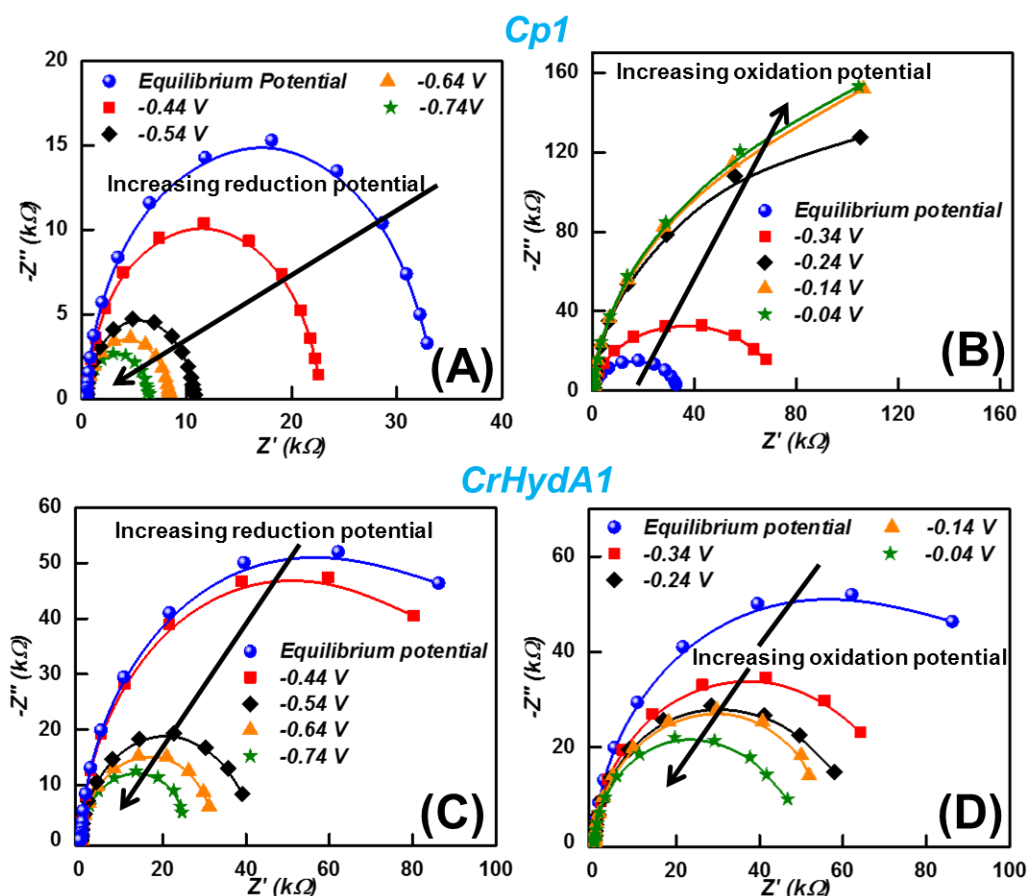


Figure 78: Impedance measurements at various potentials vs SHE: (A) at most negative potentials where CpI catalyzes H^+ reduction (black arrow shows increasing reduction potential); (B) at more positive potentials where CpI catalyzes oxidation of H_2 to H^+ (black arrow shows increasing oxidation potential); (C) at most negative potentials where CrHydA1 catalyzes H^+ reduction (black arrow shows increasing reduction potential); (D) at more positive potentials where CrHydA1 catalyzes oxidation of H_2 to H^+ (black arrow shows increasing oxidation potential). The symbols in the plots are experimental data points and the lines are the theoretical fit. This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶ Experimental conditions: pH = 7 (0.10 M phosphate buffer), T = 0 °C, rotation rate = 1000 rpm, scan rate = 20 mVs⁻¹.

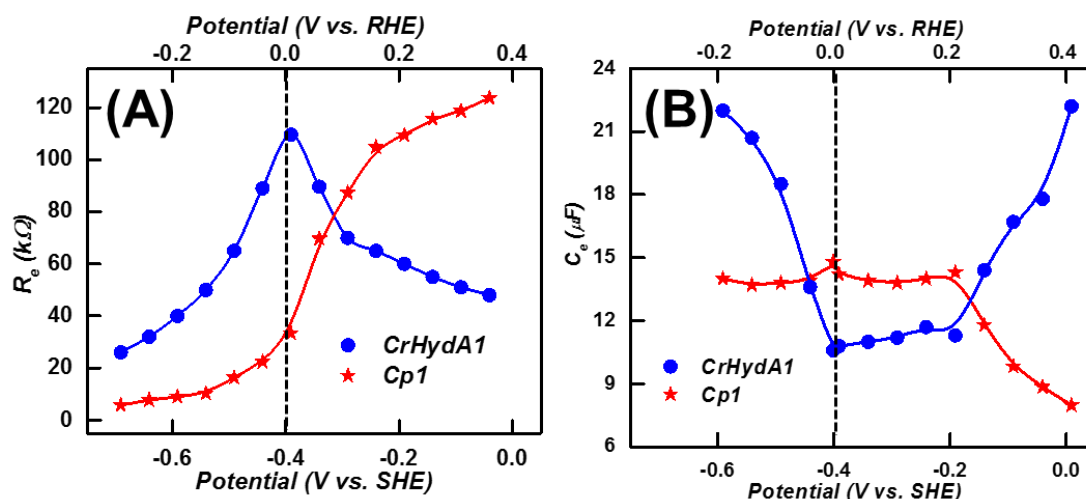


Figure 79: Potential-dependent variation of (A) R_e values and (B) C_e values for CpI and CrHydA1 on the PGE electrode. The symbols in the plots are experimental data points and the lines are included to guide the eye. The dotted line shows the equilibrium potential (proton reduction potential) with respect to RHE and SHE. This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶ Experimental conditions: pH = 7 (0.10 M phosphate buffer), T = 0 °C, rotation rate = 1000 rpm, scan rate = 20 mVs⁻¹.

As observed above, the arcs behave similarly in the proton-reduction side and for both CpI and CrHydA1, there is a corresponding fall in the value R_e with increase in reduction potential. The contrasting behaviour on the oxidizing side leads to two observations: 1. the resistance displayed by CpI when the enzyme is fully charged increases with applied overpotential but decreases for CrHydA1 and 2. the time involved in getting the enzyme to being fully charged increases for CpI and decreases for CrHydA1.

Since the two enzymes have identical H-cluster and similar protein environment surrounding the H-cluster, it is conceivable that the increase in R_e upon application of oxidizing potential seen within CpI arises from the Fe-S cluster. The Fe-S centres of CpI that are detectable by EPR have an average reduction potential of approximately -420 mV vs SHE although specific values for the two distal Fe-S centres are currently

unknown and may be more negative. Furthermore, it may be that the low-potential Fe–S clusters at the protein surface become more resistant to receiving electrons when increasingly more oxidizing overpotential is applied. The increasing resistance offered by the low-potential Fe–S cluster serving as the entry/exit could also explain the time needed for the active site of *CpI* to becoming fully charged and the increased value of that resistance with respect to *CrHydA1*. With *CrHydA1* having no Fe–S clusters forming an electron relay to the active site, the transfer of electrons across the PGE surface to the enzyme is the dominant factor and increasing overpotential on the oxidizing side ensures there is more driving force to bring about catalytic reaction.

Figure 79B shows how C_e values of the two enzymes vary with overpotential. For *CpI*, C_e remains constant and large up to an overpotential $\eta = 0.2$ and then falls. The C_e values found by fitting for *CrHydA1* show a broad and constant minimum extending between 0 and 0.2V overpotential values which then increase on either side. The fact that for *CpI* the capacitive value is maximum and constant from the most negative value of the applied potential sweep to 0.2V overpotential (on the oxidizing side) is an indication of the active site's capacitive character manifesting in that potential range. Beyond the 0.2V overpotential the capacitive behaviour decreases as the resistance offered by the Fe–S cluster increases. It may be that the resistance by the relay chain interferes with the charging process of the active site. The result of these interactions explain the voltammogram as obtained and shown in Figure 74 A. For *CrHydA1*, an decrease in the resistances of electron transfer to and fro from the active site on either side of the equilibrium potential is directly reflected in the capacitive behaviour of the active site (except in the 0–0.2V overpotential region). As the resistance decreases on either side of the equilibrium potential, the capacitive behaviour of the active site

increases. The trend suggests that for *CrHydA1*, there is room for its activity to increase furthermore with increasing overpotential. For *CrHydA1*, electron transfer rate on either side of the equilibrium potential is the rate-limiting factor.

6.4 Computation Results based on results of EIS

Both *CpI* and *CrHydA1*enzymes have the same active site: the main difference in electrocatalytic properties of *CrHydA1* and *CpI* arises from the facilitation and control of electron transfer by the Fe–S relay. For *CpI*, the fact that the internal Fe–S relay offers little resistance at potentials close to and more negative than the equilibrium potential must be a major factor in determining the catalytic *bias* that lies well in favour of H₂ evolution vs H₂ oxidation at neutral pH values. According to a recent model,²⁹ which assumes that the H₂ concentration is high enough that its binding or dissociation is not important, a key factor in determining the catalytic *bias* of an enzyme is the difference between the reduction potential of the site(s) at which electrons enter or leave the enzyme and the reduction potential of the reaction being catalysed: even a small difference (< 50 mV) can result in a large difference between oxidation and reduction rate.

Lacking a long-range FeS relay, *CrHydA1* displays no such catalytic bias; instead, as confirmed by the R_e and C_e values, the catalytic rate is limited by surface electron transfer in each direction. Hence, modelling by Butler– Volmer (BV) theory was carried out to understand the impact of overpotential on the electron transfer in each direction.

6.4.1 Modelling for CrHydA1 using Butler-Volmer theory

The Butler-Volmer equation relates the current value at a certain potential, i_E to the overpotential, η by the equation:

$$i_E = i_0 \left\{ \exp\left(\frac{-\alpha\eta F}{RT}\right) - \exp\left(\frac{(1-\alpha)\eta F}{RT}\right) \right\} \quad \text{Equation 32}$$

where i_0 is the exchange current density, η is the overpotential and R, T and F have their usual meanings. Figure 80 shows the cyclic voltammogram of CpI and CrHydA1 (black traces) to which the theoretical curves as obtained by BV are imposed as red traces calculated to show how the electrocatalytic current of CrHydA1 should vary with applied electrode potential if limited by interfacial one-electron transfers.²⁹

It is possible to calculate the theoretical values from BV by the use of i_0 as derived for the enzymes by EIS (Section 6.3.2). For CpI, the red trace deviates from the experimental result with the enzyme performing better than can be explained by electron transfer rate alone (for ~ 0.1 V overpotential on the oxidizing side and ~ 0.2 V overpotential on the reducing side). The presence of Fe–S cluster and its potential difference from the reaction at the active site creates a *bias* which shall be explored later in Section 6.4.4. For CrHydA1, the electron transfer rate obtained by BV is able to explain the experimental CV with 0.1 V overpotential on either side. It was decided to use current-potential dependencies using Marcus-Hush-Chidsey theory which takes a further factor, reorganization energy, λ , into account in addition to overpotential, η .

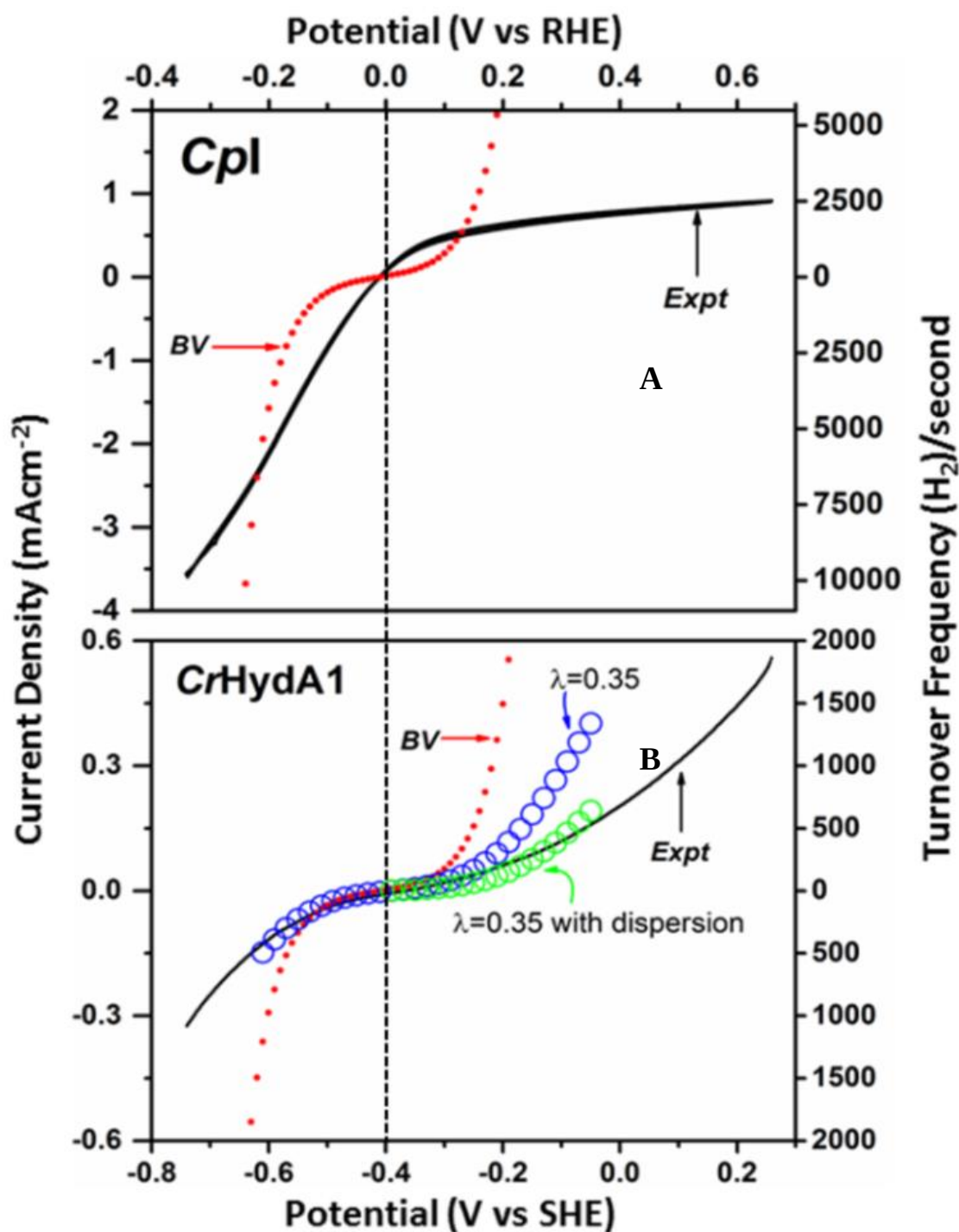


Figure 80: Analysis of voltammograms for (A) *CpI* and (B) *CrHydA1* scaled with respect to current densities and turnover frequencies. Comparisons are made in each case with current-potential dependencies calculated using a simple Butler-Volmer model (red dots). The vertical broken line marks the equilibrium potential vs RHE and SHE. For *CrHydA1*, comparisons are also shown with current-potential dependencies determined using Marcus-Hush-Chidsey theory¹²⁴ with $\lambda = 0.35$ eV (blue open circles) with some dispersion due to inhomogeneity being taken into account (green open circles). This figure is reprinted (with permission) from impedance paper by Pandey *et al.*¹⁶⁶

6.4.2 Modelling for CrHydA1 using Marcus-Hush-Chidsey (MHC) theory

The Marcus-Hush-Chidsey (MHC) model is well known in electro-analytical chemistry and the work in this thesis utilizes Equations 7 and 8 in a review by Henstridge *et al*¹²⁴ adopted by Wolfram Mathematica software:

$$f[y, \lambda] := 25 * 1000 * 96485 * 1.26e - 12 * \text{Exp}[-0.5 * 42.51 y] * \frac{\int_{-\infty}^{\infty} \frac{\text{Exp}\left[-\frac{(x - 42.51y)^2}{170.04 + \lambda}\right]}{2 \cosh\left[\frac{x}{2}\right]} [x, -10000, 10000]}{\int_{-\infty}^{\infty} \frac{\text{Exp}\left[-\frac{(x)^2}{170.04 \lambda}\right]}{2 \cosh\left[\frac{x}{2}\right]} [x, -10000, 10000]} \quad \text{Equation 33}$$

$$g[y, \lambda] := 25 * 1000 * 96485 * 1.26e - 12 * \text{Exp}[0.5 * 42.51 y] * \frac{\int_{-\infty}^{\infty} \frac{\text{Exp}\left[-\frac{(x - 42.51y)^2}{170.04 + \lambda}\right]}{2 \cosh\left[\frac{x}{2}\right]} [x, -10000, 10000]}{\int_{-\infty}^{\infty} \frac{\text{Exp}\left[-\frac{(x)^2}{170.04 \lambda}\right]}{2 \cosh\left[\frac{x}{2}\right]} [x, -10000, 10000]} \quad \text{Equation 34}$$

The MHC model allowed the evaluation of varying reorganization energies on rate constants for interfacial electron transfer. The equations derived above reduction (f) and oxidation (g) correspond to current densities (mA cm^{-2}) in which the rate constants equations by Henstridge *et al*¹²⁴ have been converted using the relationship $i = kF\Delta\Gamma$ where F = Faraday constant, A = geometric electrode area, Γ = surface coverage (Section 1.2). The two variables are ‘y’ and ‘λ’: ‘y’ is the overpotential term and equivalent to $(E - E_f^0)$ where E is the applied potential and E_f^0 is the formal potential of the $2\text{H}^+/\text{H}_2$ couple; ‘λ’ is the reorganization energy term, measured in eV. For the modelling, $k_0 = 25$, obtained from EIS experiments, and assumption $\alpha = 0.5$ were used. In Figure 80 B, the result is shown as open blue circles for $\lambda = 0.35$ which improved upon the BV result on the oxidation side by ~ 0.1 V. No effect was observed on the reduction side.

6.4.3 Modelling for CrHydA1 to investigate effect of dispersion

A further improvement in fit for CrHydA1 was achieved by introducing some dispersion in view of the complexity of protein interactions with the rough electrode surface over the entire potential range. The effect of inhomogeneity (dispersion) on interfacial electron-transfer rate constants was introduced as described in the study by Léger *et al.*¹²³ Before application of Equation 6a in that study, an assumption in the form that the exchange rate constant value (k_{ex} from EIS experiment) corresponding to k_{min} in the equation was made: thus, k_0^{max} of equation 3 of the Léger study was found by utilization of the relationship $k_{ex} = k_0^{min} = k_0^{max} \exp(-\beta d_0)$ where β is the decay constant and d_0 is the distance of closest approach. This results in the following equation, where k_{cat} is the turnover frequency, the rate at which the substrate-bound form of the active site makes and releases the product. The fit is shown in Figure 80B as open green circles by considering $\beta d_0 = 13.5$ and reorganization value of 0.35eV.

$$h(k_{cat}, \beta d_0, y, \lambda) := \frac{\left(\frac{\log\left(\frac{(0.04 + \frac{k_{cat}}{\exp[-\beta d_0]}) \cdot \exp[-0.5 + 42.51y] + (\exp[-42.51y] + 1)}{(0.04 + \frac{k_{cat}}{\exp[-\beta d_0]}) \cdot \exp[\beta d_0] \cdot \exp[-0.5 + 42.51y] + (\exp[-42.51y] + 1)}\right)}{\beta d_0} + 1 \right) \cdot 25000 \cdot 96485 \cdot 1.26 \cdot e^{-12} \cdot e^{-0.5 + 42.51y} \cdot \int \frac{E - \frac{(x - 42.51y)^2}{170.04\lambda}}{2 \cosh(\frac{x}{2})} [x, -10000, 10000]}{(\exp[-42.51y] + 1) \cdot \int \frac{E - \frac{x^2}{170.04\lambda}}{2 \cosh(\frac{x}{2})} [x, -10000, 10000]}$$

Equation 35

6.4.4 Modelling for CpI to understand bias

One avenue of research into Hyd-1 shows that it is the transfer into and out of the enzyme molecule rather than active site properties is responsible for the characteristics of overpotential and *bias* in Hyd-1.²⁷ Based on this principle, a model given by Hexter *et al.*²⁹ was used to model the CpI voltammogram as shown in Figure 81(converted to current density).

Again, Mathematica Wolfram software was utilized and results were computed using the following equation:

$$j[i_{lim}, \beta d_0, p, E, e] = \frac{i_{lim}(\exp(42.51(E - e)) - 2.008)}{(\beta d_0(\exp(42.51(E - e)) + 1))} \log \left(\frac{p \exp(0.5 * 42.51(E - e)) + (\exp(42.51(E - e)) + 1)}{(\exp(-\beta d_0) (\exp(42.51(E - e)) + 1)) + p \exp(0.5 * 42.51(E - e))} \right)$$

Equation 36

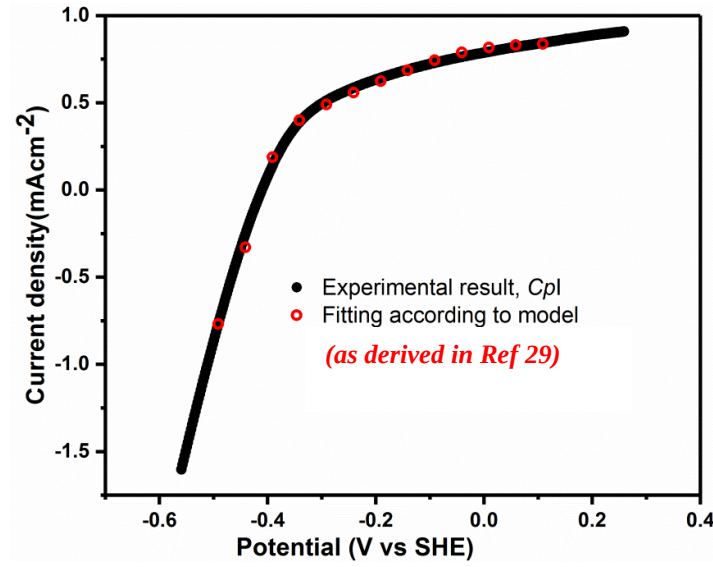


Figure 81: The best fit to the electrocatalytic profile of *CpI* given in Figure 80. The thick black trace is the voltammogram in Figure 80A (red trace) converted to current density. To understand the factors influencing bias, the model developed by Hexter *et al*²⁹ was utilized. The best fit was obtained by maintaining the variables as $i_{lim} = 0.0148$, $\beta d_0 = 13.5$, $p = 0.006$ and $e = -0.430$ V. This figure is reprinted (with permission) from impedance paper by Pandey *et al*.¹⁶⁶

The best fit was obtained as given in Figure 81 where the input function, j , depends on ' i_{lim} ', ' βd_0 ', and the quantity ' p ' (the ratio of the rate constants for the catalytic reaction compared to exchange rate constant, k_0) and have the values $i_{lim} = 0.0148$, $\beta d_0 = 13.5$, $p = 0.006$ and $e = -0.430$ V. The variations of j with ' i_{lim} ', ' βd_0 ' and the quantity ' p ' are plotted in Figure 82 to elucidate the impact of each variable on the shape of the curve. The impact of the values are as summarized in Table 7.

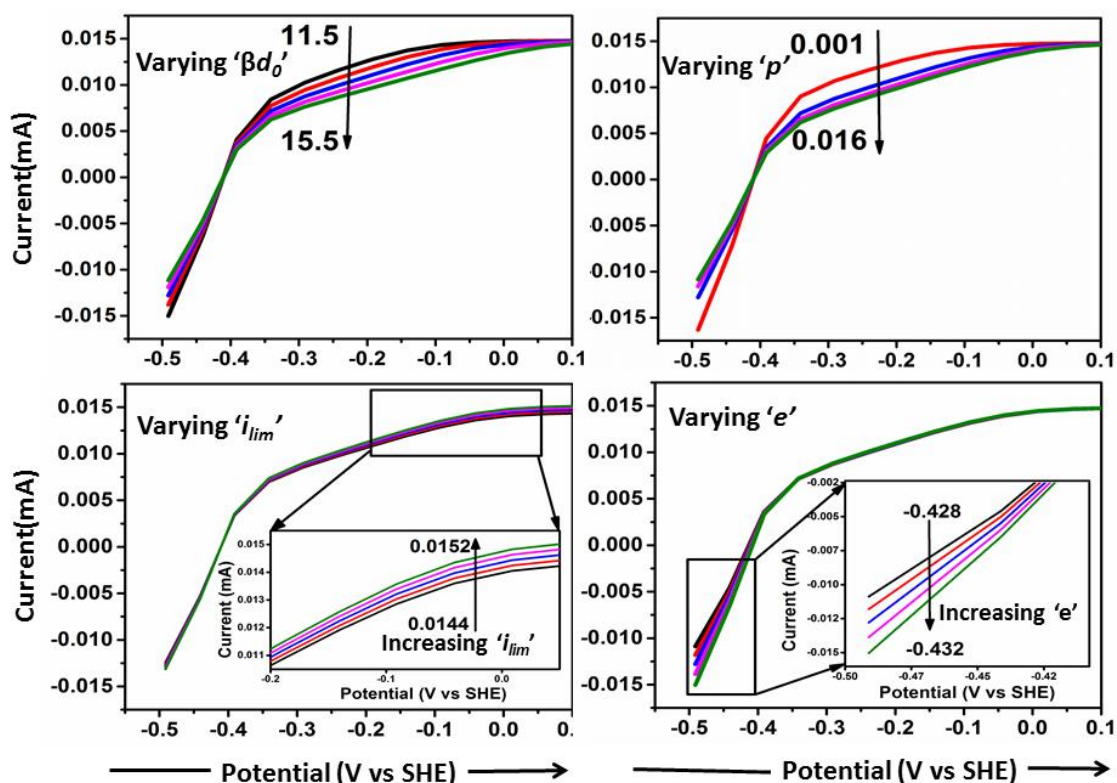


Figure 82: Plots of variations of ' j_{lim} ', ' βd_0 ' and the quantity ' p ' on the magnitude of j . The j function was modelled by input of various variables as the model developed by Hexter *et al.*²⁹ The modelling allowed the best fit to the electrocatalytic profile of *CpI* given in Figure 80 to be obtained as well as serves to clarify the impact of the variables upon the current response obtained when *CpI*-coated PGE electrode is subjected to -0.5 V to 0 V at pH = 7 (0.10 M phosphate buffer), $T = 0$ °C, rotation rate = 1000 rpm, scan rate = 20 mVs⁻¹.

Table 7: Values of the variables producing the best fit (in Figure 81) and corresponding information about catalytic *bias* of *CpI*. The best fit was obtained by maintaining the variables as $j_{lim} = 0.0148$, $\beta d_0 = 13.5$, $p = 0.006$ and $e = -0.430$ V and the information about the catalytic bias of the enzyme corresponding to the variables are given as per Hexter *et al.*²⁹ in the table.

VARIABLE	INFORMATION ABOUT THE ENZYME
$\beta d_0 = 13.5$	A larger value of βd_0 results in a more shallow increase in current with increasing overpotential. Considering a value of β as $10\text{--}12\text{nm}^{-1}$, the point of entry is calculated to be $1.125\text{--}1.35\text{nm}$.
The value of $e = -0.430$ leads to $e_2 = 2.008 > 1$ ($E_{Ox/R} < E_{eqm}$)	A value of $e_2 > 1$ ($E_{Ox/R} < E_{eqm}$) indicates a catalytic entity which is more proficient at catalysing the cathodic (reduction) reaction relative to the anodic (oxidation) reaction.
$p = 0.006$	When $p \ll 1$, the rate constants for the catalytic reaction are small compared to k_0 .

6.5 Conclusion and Perspectives

Electrochemical Impedance Spectroscopy (EIS) provided a means to obtaining valuable information of enzymes immobilized on electrodes that was not accessible using standard DC voltammetric techniques. This technique was utilized on CpI hydrogenase, a [Fe–Fe] hydrogenase with [Fe–S] clusters as part of the active site and an electron relay system, and on CrHydA1, a simple [Fe–Fe] hydrogenase with only one [Fe–S] cluster as part of the active site but not the electron relay system. The ‘*design principles*’ of these enzymes with its active site and relay system was investigated as is done in evaluation of electronic circuitries.

The real (frequency-independent) and imaginary (frequency-dependent) portions of impedance measurements were plotted as Nyquist plots. One set of results is of measurements taken at equilibrium potential while the other is of measurements taken over a range of potentials. The measurement at equilibrium potential allowed determination of the enzyme coverage and turnover frequency at the zero-current potential. Comparison of trends over a range of potentials led to the conclusion that, given the active sites of both enzymes work at the same efficiency, it is the interaction with the electrode that limits the observed turnover frequencies for CrHydA1.

The foundation of the analysis based on EIS measurements is the formation of the equivalent circuit. The arc in the Nyquist plots in the low-frequency area suggested a resistor, R , and a capacitance, C , in parallel being warranted to represent the catalytic activity of the enzyme. The resistance offered by the system at equilibrium, R_e , was then used to derive the value i_0 which should encompass a combination of long-range electron transfer (ET) efficiency and inherent catalytic proficiency of the active sites of

enzymes and could serve as the basis for selecting enzymes to be inspirations for production of biomimetic catalysts. This value reflects rate of electron flow in the absence of a driving force as does the exchange current densities from Tafel plots and Marcus electron self-exchange rates. As such, the i_0 , assigned as *electrocatalytic exchange constant*, values of different enzymes can be utilized to express their efficiency: a stumbling block would be the requirement to know the exact size of the film, *i.e.*, electrocatalytic coverage of the enzyme. Bode plots provide a means for measuring the electrocatalytic coverage of the enzyme by manifestation of k_{ex} , a measure of the inherent property of the enzyme. This value is obtained when the frequency of the applied signal resonates with the natural ‘idle’ flow of electrons. Not only does this impart information about the ease with which electrons flow within the system but, in combination with i_0 value, allows the calculation of value of electrocatalytic coverage which is not obtainable with PFE under conditions of catalysis. Based on the i_0 value corrected to negate the impact of electrocatalytic coverage, it was found that CpI is three times more efficient than CrHydA1.

Nyquist plots over a range potentials highlights differences in behaviour of CpI and CrHydA1 and moreover, explains the behaviour observed in their cyclic voltammograms obtained by PFE. For CrHydA1, the ability to carry out oxidation and reduction is enabled as a result of direct interaction of the H-cluster with the electrons that it obtains from the surface. The full activity of the active site is *not* realised in the potential range examined. The lowest activity is observed at the equilibrium potential where both the R_e and C_e values are at their lowest. The R_e values then fall with increasing overpotential while C_e values increase except in the 0–0.2V overpotential region. The activity of the active site seems to be its lowest in that range (although not

reflected in CVs) and requires a massive overpotential to *start* functioning, a reflection, in all probability of reorganization energies and other factors which are *not* reflected in CVs.

The interaction of the H-cluster with the electrons that it obtains from the surface was analysed first by application of Butler-Volmer (BV) theory which was able to explain the experimental CV with 0.1 V overpotential on either side. Additional modelling with Marcus-Hush-Chidsey (MHC) theory allowed reorganization energy, λ , to be determined as a low value, thus asserting the optimization of the H-cluster by Nature. Its inability to perform at its optimum must arise from other factors such as the lack of the electron relay which impact the electron transfer that occurs to the active site from the electrode-the inference.

For *CpI*, C_e remains constant and large up to an overpotential $\eta = 0.2$ and then falls which is exactly matched to the activity of the oxidation levelling off beyond $\eta = 0.2$. There is a corresponding trend by R_e . However, on the reduction side, the constant and full activity of the active site (constant C_e) is manifested by increase in activity in the CVs. The R_e is a constant on the reduction side: thus, the behaviour in the CV is direct reflection of the increased driving force being applied. The low-potential of Fe-S centres of *CpI* ensures that it manifests the lowest resistance and the driving force may exert its full impact. The specific values for the two distal Fe-S centres are currently unknown and may be more negative than the EPR-detected values of -420mV for the remaining Fe-S clusters. The potentials of the [Fe-S] clusters have another function: it explains the *bias* observed in *CpI* and modelling allows us to learn more about the enzyme.

Chapter 7: Concluding Deliberations

The aim of this thesis was to study the catalytic properties of some [Fe-S] cluster containing enzymes. Four different enzymes all unified by containing [Fe-S] clusters, either as an electron relay component or as part of their active sites, were studied. It was considered that protein film electrochemistry (PFE), along with some other complementary techniques, would be the best technique to utilize in carrying out the study. This chapter serves to unify the findings in terms of the [Fe-S] cluster systems that the enzymes contain.

Enzymes involved in redox catalysis usually contain electron-transferring constituents within them or their active sites are located within tunnelling distances of the surfaces. This allows electrons needed/generated from the reactions that they catalyse to transfer easily between the enzymes and their physiological redox partners. One such electron – transferring constituent is the [Fe-S] cluster, generally found within electron tunnelling distance (12-14 Å) from another, from the active site and from the enzyme surface. Within such distance, the rates of electron transfers are dependent on the redox environment of the electron donor and acceptor centres and the reorganization energies of the two. The rates are so fast that the rates of catalysis is generally a reflection on the efficiency of the active site. The [Fe-S] clusters are also involved in many other functions, e.g., in detecting iron or oxidative stresses and in gene expression. However, for the purpose of this thesis, we restricted our study to enzymes containing [Fe-S] clusters which are known or suspected of serving electron-transferring purpose.

The technique utilized throughout this thesis is ‘Protein Film Electrochemistry’ - a suite of dynamic electrochemical techniques which are generally utilized to interrogate enzymes with redox active centres. Generally, chronoamperometry and cyclic

voltammetry experiments are used which allow the examination of the reactions undertaken by the enzymes in terms of the oxidation levels of the active sites within the enzymes. The oxidation levels are altered by means of potentiostats which apply the desired DC potential. In this thesis, impedance experiments are utilized successfully for the interrogation of enzymes which are based on the application of AC voltages. Thus, an AC technique is now added to the portfolio of protein film electrochemistry.

One of the first enzymes examined within this study is Hyd-1 from *E. coli* which is known to contain a relay of three [Fe-S] clusters in its small subunit. Although the property being examined was that of a proline residue in the outer coordination sphere of the bimetallic centre making the active site, it was imperative to make experimental runs to ensure that the catalytic properties being displayed were due to changes at the active site and due to inadvertent effects on the [Fe-S] clusters. To determine the role of the proline, replacement of the residue was made with alanine, a neutral molecule which would introduce flexibility to the arginine residue that is held over the bimetallic centre in Hyd-1 but allows arginine residue to maintain the same distance from the bimetallic centre as WT (as measured by Pymol software). Protein film electrochemistry, crystallization results and differential scanning calorimetry proved the effect of proline was limited, structurally, to maintaining the rigidity of the active site though the effect was not so significant— a fact supported by the fact that other residues are found in nature to occupy the position in the protein sequence. Because the enzyme contains [Fe-S] clusters, it was imperative to carry out additional experiments, *e.g.*, O₂ tolerance and onset potential experiments, to ensure that the effects were not due to inadvertent effects on the clusters from the introduced mutation. Thus, decreased activity of the mutated sample could be reasonably assigned to changes at the active site, *e.g.*, through

loss in nickel incorporation or unfavourable H_2 activation steps. This enables ‘design principles’ of biomimetic enzymes *Sulfurospirillum multivorans* which had not been investigated successfully on an electrode before. A recent structural study revealed that two [Fe–S] clusters are present within electron tunnelling distances from the surface and the active site. This proved to be important as it enabled PFE experiments with Pyrolytic Graphite Edge (PGE) electrodes to be successful – it was possible to show that CO^{III} is accessible at highly positive values (–60 mV to 40 mV), KCN is indeed a reversible inhibitor and CO is an inhibitor in the absence of $(NH_4)_2SO_4$ in the electrolytic solution. Thus PFE is an excellent technique for interrogating enzymes with [Fe–S] clusters within tunnelling distance of the enzyme surface – in this case, it may have opened a new avenue of research into explaining why and how $(NH_4)_2SO_4$ serves to increase the activity of the enzyme.

The third enzyme, CODH, not only contains [Fe–S] cluster as part of an electron relay system but contains a modified cluster as part of the active site. The modification comes from Ni-incorporation. The Ni atom and one of the displaced Fe of the cluster is thought to work in conjunction to bring about CO and CO_2 interconversion. The O_2 sensitivity of the enzyme was investigated and it was possible to show *via* PFE that the damage to the enzyme by O_2 is potential dependent. There may be proof that the O_2 damage may be limited by the enzyme at high potential whereby the enzyme forms the C_{ox} to protect itself-this possibility at high potential is probably the result of the formation of a hypothetical state C'_{ox} which is accessible at high potential. Another interesting aspect is that low quantities of O_2 brings about a state of the enzyme which is unable to oxidize beyond –0.4 vs SHE which may be related to the redox potentials of [Fe–S] clusters of

the electron relay. Experiments showed that the maximal site of O₂ attack is the C-cluster, the active site with [Fe–S] cluster as a structural component.

It has been found that CODH behaves similarly with H₂O₂ as with O₂. Hydroxylamine, NH₂OH, which is a structural analogue of H₂O₂ has been reported as a substrate though the initial results of this report indicate it as a reversible inhibitor. This inhibition is potential dependent and 1st order with respect to the concentration of CO and NH₂OH. There is now a strong body of work to show that the electronegative elements sulfur, oxygen^{103a, 103b} and nitrogen (this work) all seem to bring about inhibition of a state accessible to the enzyme at high potential: this may possibly be resolved by EPR, an avenue of research which may prove valuable in understanding the ‘design principles’ of a CODH–based biomimetic, an important scientific and industrial quest.

Impedance experiments apply AC voltages and was utilized in PFE in order to enable the study of enzymes. Contrary to the existing techniques available *via* PFE, it allowed the calculation of electroactive coverage during catalysis. By contrasting two enzymes, CrHydA1 and CpI, one without an [Fe–S] electron relay system and one with, this technique allowed quantitative comparison of the two enzymes as is possible in irreversible systems—it was found out that CpI is two times more efficient than CrHydA1. In all probability the [Fe–S] cluster containing electron relay system allows CpI to function better than CrHydA1. In CrHydA1, the rates of electron transfer from the electrode surface into the enzyme were found to be rate-limiting—it was possible to model the voltammograms quite reasonably with Butler-Volmer and Marcus theories. The [Fe–S] cluster containing electron relay system of CpI is branched to allow better contact with the electrode surface and, in all probability, its superior functioning.

The aims of thesis were thus met as it proved PFE a powerful tool, especially in examining the catalytic properties of four different [Fe-S] cluster containing enzymes. It was possible to derive valuable information regarding them and opened up further avenues of possible research which may prove to be both industrially and scientifically significant.

Chapter 8: Materials and Methods

8.1 Electrochemical set-up

For protein film electrochemistry (PFE) experiments, the three-electrode system was used with Pyrolytic Graphite Edge (PGE) electrodes, constructed in-house as described in Armstrong *et al.*⁵⁰ serving as the working electrode (WE). In all experiments, a platinum wire was used as the counter electrode (CE) to prevent significant drawing of current through the reference electrode (RE), a saturated calomel electrode (SCE). The electrochemical cell housed a Luggin capillary side arm filled with 0.1 M NaCl (Fisher Scientific, 99.7% Analytical Grade) for immersion of the RE– the fitting was ensured to be airtight. This side-arm was maintained at room temperature as a non-isothermal reference, with conversion to the standard hydrogen electrode (SHE) scale using $E_{\text{SHE}} = E_{\text{SCE}} + 241\text{mV}$ at 25 °C¹²² and to the reversible hydrogen electrode (RHE) scale using the correction $E_{\text{RHE}} = E_{\text{SHE}} - 0.059\text{pH}$,¹²² in full accordance with zero-current potentials observed in experiments, for reporting purposes. The electrochemical cell was water-jacketed for thermostating purposes and rubber septums were used to seal various side-arms (of small diameters and lengths) to make the system gas-tight; needles through the septum provided inlet and outlet provisions for any gasflow desired. The entire set-up was housed in anaerobic gloveboxes as the enzymes being handled were all oxygen-sensitive. All the gloveboxes were maintained under an N₂ atmosphere (Belle Technologies or MBraun or Vac Atmospheres (O₂ < 3 ppm)) and potential control was achieved by use of potentiostats. Measurements of *E. coli* Hyd-1 (both WT and P508A mutant) parameters with PFE were carried out using a PGSTAT128N Autolab potentiostat controlled by Nova software (EcoChemie). Autolab PGSTAT30 and PGSTAT128N were used to carry out PceA dehalogenase experiments while CODH experiments were carried out using Autolab PGSTAT20. All Electrochemical Impedance Spectroscopy (EIS) electrochemical experiments with CpI and CrHydA1

were carried out using an Ivium CompactStat E800 workstation equipped with a frequency response analyser (Ivium Soft 2.4).

The enzyme films of *E. Coli* Hyd-1 on PGE required special treatment before utilization— the process is known as ‘activation’ and is outlined below:

For each experiment, the PGE electrode surface was roughened by sanding with P400 grade Tufbak Durite paper from Hookit™. In each case, 1-2µl of Hyd-1 enzyme (both WT and P508A variant) was pipetted up and down onto the electrode surface several times and allowed to dry for ~30 s before rinsing with purified Milli-Q (MQ) water. Since Hyd-1 ‘switches off’ to form oxidized inactive states upon exposure to air during prep, it needs to be converted back to the active state before characterization by PFE can be carried out. The time required for full activation varies and it was carried out by repeatedly poisoning the electrode at -0.659 V vs SHE for 300 s then monitoring the H_2 oxidation activity at 0 V vs SHE for 60 s until the activity level stabilized (judged to occur when voltammograms overlapped or decreased). All other enzymes utilized in this thesis were applied to the electrode as described above but were allowed to dry for ~1 min without any subsequent rinsing. No activation procedure was required. The CODH enzyme was mixed with polymyxin β -sulfate, in the ratio of 1:3 by concentration, before being spotted onto PGE electrodes.

To obtain a higher coverage of enzyme for the production of detectable levels of formate and ammonia formed by CODH under defined conditions, the enzyme was covalently linked to the surface of the electrode as follows: a suspension of multi-walled carbon nanotubes (MWCTs) in dimethyl formamide (DMF) ((15 µL of 1 mg/mL) was

pipetted onto the PGE surface and left to dry overnight. The MWCT–PGE electrode surface was then treated with a solution of 1-pyrenebutyric acid (Py) in DMF (20 μ L aliquot of a 10 mM solution) and allowed to stand for 1 h. The modified electrode was then washed with MQ water to remove any free Py and then taken into the glovebox for the next stages of modification. A mixture comprising 5 μ L of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.4 M) and 5 μ L of N-hydroxy sulfosuccinimide (NHSS) (0.1 M) was spotted onto the modified PGE surface and after 20 min, measured quantity of CODH I_{Ch} was pipetted on and left for 40 min with the electrode covered by an Eppendorf tube to preserve humidity. Multi-walled carbon nanotubes (MWCT, carbon >90% (trace metal basis)), 1-pyrenebutyric acid (Py), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy sulfosuccinimide (NHSS) were all purchased from Sigma-Aldrich and used for this modification. Dimethyl formamide (DMF) was purchased from Acros Organics (99.8+%)

8.2 Fabrication of Pyrolytic Graphite Edge (PGE) electrodes

Pyrolytic graphite edge (PGE) electrodes were fabricated in-house.⁵⁰ The electrodes consist of small pieces of pyrolytic graphites (Momentive; it was shaped by PTCL workshop, University of Oxford, UK) embedded in epoxy resin (Loctite EA9466, Henkel) built upon Teflon casings containing metal contact rods. The epoxy resin had been mixed with 1 part hardener by mass and gently poured into ~1.5 turns of Scotch magic tape around the edge of the Teflon casing for the embedding. The metal rod makes electrical connection with the pyrolytic graphite edge by application of silver glue (Agar Scientific) and silver loaded epoxy adhesive (RS) and it is ensured that the whole electrode did not exert more than 10 Ω resistance (as measured by a digital multimeter) with 4-7 Ω being considered ideal. The graphite was oriented so that the

basal planes were perpendicular to the surface of the electrode shaft. The graphite electrodes were of approximately 3-9 mm² area. For improved signal-to-noise results, as was required for the formate and ammonia detection experiments, the PGE electrodes were modified using a multi-walled carbon nanotube (MWNT)/activated 1-pyrenebutyric acid system as outlined by Krishnan *et al*¹²⁰ and described in detail above.

8.3 Buffers and Solutions Utilized in PFE Measurements

All buffers were made with MQ water (18 MΩ cm) which was obtained via a Millipore Elix® 5 system and further purified through a Milli-Q system with a 0.22 μm Millex® syringe-driven filter unit (Millipore). They were made to the desired pH (monitored with Mettler Toledo SevenEasy pH meter) using conc. HCl or NaOH (Fisher Scientific, Analytical Reagent Grade) at the working temperature of the designed experiment.

MES Buffer: MES, (2-(N-morpholino) ethanesulphonic acid, of pKa 6.10 (Melford Laboratories Ltd., >99%) was used to make 200 mM solutions with purified MQ water and then titrated to the desired pH for PFE studies of CODH.

TRIS Buffer: Tris buffer solutions (100 mM) for PceA dehalogenase experiments were prepared by dissolving Tris (Tris(hydroxymethyl) aminomethane, Fisher, 99.8%) in purified MQ water. Then, (NH₄)₂SO₄ (Fisher, 99.8%) was added to a final concentration of 4 mM and the resultant mixture titrated to the desired pH. For use in comparison studies, (NH₄)₂SO₄ was not added in order to provide 'TRIS only' buffers.

Phosphate Buffer: Phosphate buffers at pH 7 (100 mM) were used for Cpl and CrHydA1 impedance and PFE experiments. The buffer was prepared to the desired pH by titrating individually prepared solutions of 100 mM monobasic (Sigma Aldrich, ≥98%) and 100 mM dibasic sodium phosphate (Acros Organics: 99%). For hydrogenase

solution assays, methylene blue (Sigma-Aldrich) was added to a final concentration of 25 μ M in a (similarly prepared) 50 mM potassium phosphate buffer solution (pH 6).

Mixed Hydrogenase buffer: A mixed buffer solution was used to carry out experiments involving Hyd-1(both WT and P508A variant). It was prepared as a solution containing 100 mM NaCl (Fisher Scientific 99.7% Analytical Grade), 15 mM anhydrous sodium acetate (Fisher Scientific Analytical Grade, pKa 4.76), 15 mM CHES (2-(cyclohexylamino)ethanesulfonic acid, Melford, >99%, pKa 9.30), 15 mM HEPES (N-(2-hydroxyethyl)-1-piperazine-N-(2-ethanesulfonic acid, Melford, >99.5%, pKa 7.55), 15 mM MES (2-(N-morpholino)ethanesulfonic acid, Melford, >99%, pKa 6.10) and 15 mM TAPS (N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, Melford, >99% pKa, 8.40).

CO-saturated TRIS/ phosphate buffer: A CO cylinder (CP-grade, BOC) was fitted to the glove box and used to saturate the relevant buffer contained in a sealed glass vial inside the glovebox. The vial had been fitted with a rubber septum into which was inserted needles to provide a gas inlet and outlet. Bubbling the gas through the buffer solution for 20 minutes ensured saturation with CO. Generally, 1-5 ml of the solution were injected into the electrochemical cell for an observed effect.

O₂-saturated MES/ mixed Hydrogenase buffer: A similar method to that for the preparation of CO-saturated buffer, was used for O₂-saturated buffer; an O₂ cylinder (BOC) was fitted to the glove box. Similar bubbling for 20 minutes ensured saturation of the buffer with O₂ (~ 1.3 mM at 20 °C). Generally, 1-2 ml of solution was injected into the electrochemical cell for an observed effect.

KCN solution: Measured quantities of KCN (Fisher, Analytical reagent grade) were taken into the glove box and dissolved in MQ water to yield required concentrations. This method was preferred to the more traditional methods of purging the solution and

then taking into the glovebox. It served to save time and avoid complications which might arise from (accidental) insufficient purging. This was also done when making hydroxylamine solution (see below). In addition, KCN solution subjected to purging may lose content as HCN. Measured quantities (generally, 1-2 ml of the solution) were injected into the electrochemical cell to give a desired final concentration.

Hydroxylamine solution: Measured quantities of hydroxylamine-HCl(Sigma-Aldrich, 99%) were taken into the glove box and dissolved in MQ water to yield required concentrations. For use, measured quantities (generally, 1-5ml of the solution) were injected into the electrochemical cell.

Hydrogen peroxide solution: Measured quantities of hydrogen peroxide (30% w/w from Sigma-Aldrich) were made up to volume with MQ water and purged with in-house N₂ for 0.5 hr before being taken into the glovebox for use.

PCE, TCE, *cis*-DCE, DBE and DCP solutions (in ethanol): Ethanol was added to measured quantities of the substrates (PCE, TCE, *cis*-DCE, DBE and DCP) in glass vials such that the concentration was 8 mM. The substrates were all purchased from Sigma: PCE = 99.5% , TCE = 99.5%, *cis*-DCE = 97%, DBE = 98% and DCP =98%. The solution was then purged with in-house N₂ for 0.5 hr before being taken into the glovebox for use. The substrate stock solution was injected into the electrochemical cell to yield a final concentration of 800 μ M, unless otherwise required.

8.4 Injection Techniques for Introduction of Substrates and Inhibitors

Many of the experiments required either the addition of inhibitors or substrates by injection. For CODH experiments, O₂-saturated buffers were prepared and a measured quantity, depending on the experiment, taken into a syringe; the needle was then

inserted through one of the rubber septums sealing the side-arms of the electrochemical cell, and the buffer was injected. Injection of the solution was carried out very carefully by placing the needle far enough such that the solution dropped into the cell solution but not so far that the added solution was dispersed by the rotating electrode. A slow pace ensured that all of the injected solution reached the cell solution but it was maintained at a rate fast enough so that the addition of O₂-saturated buffer was not dispersed across a time frame that was reflected in the voltammogram or chronoamperometry graph. Inhibitions by CO-saturated buffers (for *Cpl* and *CrHydA1* impedance measurements and *PceA* dehalogenase measurements), KCN solutions (for CODH and *PceA* studies) and O₂-saturated buffers, hydroxylamine and hydrogen peroxide solutions (for CODH PFE measurements) were carried out similarly. When possible, injections took place in the instrumental ‘dead time’ between the end of one cycle of CV measurements and the start of the next. Some substrates such as PCE, TCE, *cis*-DCE, DBE and DCP were injected in the same way into the electrochemical cell.

8.5 Molecular Biology Methods

The P508A mutation was introduced into the chromosome of *E. coli* by site-directed mutagenesis (SDM). The mutation was made as a *pMAK_hyaB_P508A* plasmid by Gibson Assembly¹⁴⁰ which was subsequently transformed into competent *E. coli* DH5 α cells. The mutation was confirmed by DNA sequencing by Source BioScience. The codon change was then transferred from the plasmid to the chromosome of MC4100-derived K-12 strain FTH004 as per the *pMAK* protocol detailed by Hamilton *et al*¹²⁷ and again, the whole gene integrity was confirmed by DNA sequencing by Source BioScience. The various bacterial strains utilized in this work are given in Table 8.

Table 8: Bacterial strains used for the production of *E. coli* Hyd-1 (both WT and P508A variant).

ORGANISM	RELEVANT CHARACTERISTICS	SOURCE
<i>E.coli</i> DH5 α	<i>recA1 endA1 hsdR17 lacZAM15</i>	New England Biolabs
<i>E.coli</i> K-12 FTH004	<i>recA⁺ hyaA^{his}</i> FTH004 contains a modified HyaA protein bearing a hexa-histidine tag at the C-terminus	Dubini <i>et al</i> ¹³⁷
<i>E.coli</i> Hyd_1_HyaB_P508A	<i>recA⁺ hyaA^{his}</i> FTH004 (Hyd_1_HyaB_P508A) P508A variant strain. Contains a modified HyaA protein bearing a hexa-histidine tag at the C-terminus. Strain is produced by codon change on chromosome of <i>E.coli</i> K-12 FTH004 strain using <i>pMAK-hyaB-P508A</i> plasmid.	STAI (this work)

8.5.1 Initial PCR for Gibson Assembly

The initial PCR utilized to create the two mutation-bearing *ds*-DNA segments for Gibson assembly were carried out with the reagents and conditions as per manufacturer's instructions outlined in Table 9 and Table 10; primer sequences are given in Table 11.

Table 9: Reagents for the PCR. Two vials were set to run on PCR: vial 1 contained *hyaB_P508A_F* primer with *pMAK Rev* primer to yield 5896kb *ds*-DNA segments and vial 2 contained *hyaB_P508A_R* primer with *pMAK Fwd* primer to yield 2568kb *ds*-DNA segments. The sequence of the primers are given in Table 11.

REAGENT	VOLUME (μ L)
<i>pMAK705</i> Template	0.5
Q5 High Fidelity Polymerase (NEB)	0.5
DNTPs (10 mM, NEB)	1
Mutagenic Primer (10 mM, Invitrogen)	2.5
Gibson Primer (10 mM, Invitrogen)	2.5
Reaction Buffer (NEB)	10
G/C Enhancer (NEB)	10
Water	23
Total	50

Table 10: PCR cycling parameters

DESCRIPTION	TEMPERATURE (°C)	TIME (S)	REPEATS
Initial denaturation of DNA	98	120	
Denaturation of DNA	98	30	Repeat x35
Annealing of primer to DNA	55	60	
Extension of chain	72	165	
Final extension	72	18000	

Table 11: Primers used in initial PCR for Gibson Assembly.

PRIMER	FUNCTION	
hyaB_P508A_F	Mutagenic forwards primer to create P508A mutation	5'-GGT TTT ACC GAA GCG GCC CGC GGG GCG TTA GGC CAC TGG-3'
hyaB_P508A_R	Mutagenic reverse primer to create P508A mutation	5'-CCA GTG GCC TAA CGC CCC GCG GGC CGC TTC GGT AAA ACC-3'
pMAK Fwd	Counterpart primers for Gibson Assembly	5'-CGG TCC AAT GAT CGA AGT TAG GCT GGT AAG AGC CG-3'
pMAK Rev	Counterpart primers for Gibson Assembly	5'-CCA GTG GCC TAA CGC CCC GCG <u>GGC</u> CGC TTC GGT AAA ACC-3'
HyaB- F	Amplification of the HyaB gene from chromosomal DNA for colony PCR	TATTGAATTTCGCGCCATCATTACTTAC ATGGTGACCTTTG
HyaB- R	Amplification of the HyaB gene from chromosomal DNA for colony PCR	TATTGAATTCCGCGCGCAAAAATAGCG TACTGGCTGTGTTTCGC

DpnI was used to digest methylated DNA present in the PCR by subjecting the reaction mixtures to incubation with 0.5 μ L of DpnI (NEB) for 9 hours at 37 °C before the enzyme was denatured at 50°C for 1 hour. This, in combination with the low amount of template used in the initial PCR reaction, increases the probability that the transformation carried out in later stages occur by mutated plasmid only. After DpnI digestion, DNA electrophoresis was subsequently performed in order to confirm that the linear *ds*-DNA sequences were of the correct length by comparison to 1 kb DNA Ladder (NEB). A 2% w/v agarose (Melford, Molecular Biology Grade) gel in TAE buffer (Appendix 9) was prepared. Loading dye (6X, NEB) was added to the samples before they were electrophoresed at 100 V for 240 minutes by LKB Bromma 2197

Power Supply to ensure clear separations of the bands. Illumination in UV light by a UVP White/2UV™ Trans-illuminator allowed the bands that corresponded to the correct size (5896 bp and 2568 bp) to be visualized and extracted from the gel using a QIAquick Gel extraction kit (Qiagen) according to the manufacturer's instructions and submitted for sequencing. Sequencing was carried out with suitable primers by Source Bioscience.

8.5.2 Gibson Assembly

Once the linear fragments were confirmed to contain the mutation, they were added to a Master Mix (NEB) that contained the polymerase, 5'-exonuclease and ligase in the appropriate ratio. The utilized quantities of the Master Mix and the two *ds*-DNA segments for this reaction are shown in Table 12; the reaction was allowed to proceed for four hours at 50 °C.

Table 12: Reagents utilized for the Gibson Assembly procedure to create *pMAK_hyaB_P508A* (the P508A-containing plasmid).

REAGENT	VOLUME (μ L)
Gibson Assembly Master Mix (NEB)	3
DNA fragment 1	1
DNA fragment 2	1
Water	1
Total	6

Of the 6 μ L reaction mixture from Gibson Assembly (Table 12), 3 μ L was taken and transformed into 100 μ L chemically competent DH5 α cells by heat shock treatment (Appendix 6). After transformation, the cells were plated on LB/Cam at 30 °C for 48 hours. Any bacterial colonies were picked into 5 mL of LB/Cam and grown in a shaking incubator at 200 rpm and 30 °C overnight. A miniprep kit (QIAprep Spin Miniprep Kit, Qiagen) was used to isolate the DNA from the cultures as per the

manufacturer's instructions; sequencing was carried out by Source Bioscience in order to confirm the presence of the mutation.

8.5.3 pMAK Cycle and Colony PCR

The pMAK Cycle: In order to commence the cycle, FTH004 cells were made competent by the RbCl method (Appendix 5). The pMAK_705 plasmid bearing the P508A mutation was then chemically transformed into these FTH004 cells as per the protocol described by Evans *et al*⁴⁴ which is based on theory outlined by Hamilton *et al*.¹²⁷ The FTH004 cells contain an engineered HyaA protein with a His6 affinity tag at its C-terminus.⁵⁷ The cells were plated on LB/Cam¹ agar plates from which (after 24 hrs) a single colony was picked and put into 5 mL LB/Cam solution and allowed to grow overnight in a shaking incubator set to 200rpm at 30°C. An aliquot of 100 µL of this culture was then diluted with 900 µL MQ water to create a 10⁻¹ dilution. This process was repeated to create serial dilutions from 10⁻¹ to 10⁻⁷. The 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions which were subsequently plated, in duplicate, onto LB/Cam plates and grown at 44 °C overnight whilst the 10⁻⁷ dilution was plated onto LB/Cam and grown at 30 °C overnight in order to act as a control. Growth overnight on the 30°C plate confirmed the integrity of the process and the cycle was continued with plates made from the rest of the dilutions.

3 x 10 mL of LB/Cam media tubes were then prepared and four colonies from each of the serial dilution plates were picked into a tube per dilution. These were grown overnight at 30 °C (200 rpm) before a 10 µL subculture from each tube was placed into a fresh 10 mL LB/Cam solution and allowed to grow overnight. A loop-full of each

¹ Stock solutions of chloramphenicol of 25mg/ml in 100% ethanol were prepared and diluted 1000 times for working concentrations of 25µg/ml to be used in LB medium and agar plates.

culture was streaked on an LB/Cam plate (*i.e.* 3 plates in total) and grown overnight at 30 °C. A single colony was then picked from each plate and used to inoculate 10 mL of LB-only broth in duplicate (*i.e.* 6 x 10 mL samples total) at 44°C overnight. A loop-full of each culture was then streaked to single colonies on LB-only plates which were then grown overnight at 44 °C. An LB/Cam plate and an LB-only plate were then prepared and 52 individual colonies were picked from the 6 streaked plates onto both the LB only and LB/Cam plate simultaneously. These plates were grown overnight at 30 °C in order to determine which of the colonies were Cam-sensitive and hence did not have free plasmid in them. Corresponding colonies on the LB plate were then chosen for the Colony PCR stage.

Colony PCR: A master mix was prepared and 14 colonies from the *pMAK* cycle was screened simultaneously. The colonies were individually suspended in 100 µL of sterile MQ water and vortexed: 4µL of the suspension was combined with 50µL of the master mix. Reagents for the master mix and conditions for colony PCR are shown in Table 13: and Table 14 respectively.

Table 13: Reagents for Colony PCR Master Mix

REAGENT	AMOUNT (IN µL)
MQ water	591.5
Taq Buffer (Invitrogen)	75
W1 detergent (Invitrogen)	35
MgCl ₂ (50 mM stock, Invitrogen)	21
HyaB-F (100 mM, Invitrogen)	7
HyaB-R (100 mM, Invitrogen)	7
Taq Polymerase (Invitrogen)	7
dNTPs (2.5mM stock, Invitrogen)	7

Table 14: Conditions for colony PCR

DESCRIPTION	TEMPERATURE (°C)	TIME (s)	REPEATS
Initial denaturation of DNA	95	300	
Denaturation of DNA	95	30	Repeat x35
Annealing of primer to DNA	50	30	
Extension of chain	72	600	
Final extension	72	420	

8.5.4 Isolation and Purification of enzymes

The isolation of hydrogenase enzymes (both WT and its P508A variant) was carried out according to the methods outlined in Lukey *et al.*⁵⁷ The colony confirmed to contain the P508A mutation was inoculated into 5 mL of LB and grown overnight to create a liquid starter. This was then added to 6 L LB containing 0.4% (w/v) sodium fumarate (Alfa Aesar, 98%) and 0.5% (v/v) glycerol (Fisher Scientific Laboratory reagent grade), and then grown anaerobically at 37 °C for 16 hours. The subsequent steps were then conducted at 4 °C. The bacteria were then collected by centrifugation at 5000 rpm for 12 minutes before being resuspended in Buffer 1 (Appendix 9), which had 50 µg mL⁻¹ lysozyme (Sigma Aldrich, 10000U/mg) and 10 µg mL⁻¹ DNAase (Sigma Aldrich, >>400Kunits/mg protein) added to it. This resuspension was then passed twice through a Constant Cell Disruption System at 20 kPsi before being centrifuged at 3500 rpm for 30 minutes. The membrane fraction was then isolated by ultracentrifugation at 45000 rpm for 1 hour and any unlysed cells were passed through the Cell Disruption System once again at 25 kPsi and centrifuged at 3500 rpm for 30 minutes. This fraction was then also collected and centrifuged at 45000 rpm as before. The two pelleted membrane fractions were pooled and resuspended in Buffer 2 (Appendix 9) before being left to stir overnight for homogenization.

In order to complete the solubilisation of the membranes, 30% Triton X-100 solution was added (4 mL per 6 L bacterial culture) and the suspension stirred for 40 minutes to overcome the strong interaction between α -helix and lipid tail binding. After that, the suspension was ultracentrifuged at 45000 rpm for 1 hour in order to remove any insolubilized components. A 5mL HisTrap HP column (GE Healthcare) was then equilibrated using 50 mL of Buffer A (Appendix 9). The solubilised membranes were then loaded onto the column and washed with a further 100 mL of Buffer A. The protein was then eluted from the column using an increasing linear gradient of imidazole. This was achieved by increasing the amount of Buffer B (Appendix 9), relative to Buffer A(Appendix 9), from 0 to 100% over 27 mL. Fifty 1 mL fractions were then collected. 10 μ L was taken from six fractions, selected by the Äkta's UV traces, to confirm the presence of matured enzyme (by SDS Page) before the enzyme containing fractions were then pooled and concentrated using a 50 kDa Vivaspinn 20 centrifugal concentrator at 3500 rpm. A desalting column (GE Healthcare) was then prepared by washing with five column volumes of Buffer C (Appendix 9) and the concentrate was loaded onto it. The protein was then eluted with Buffer C: 10 μ L of the resulting solution was taken to calculate the final concentration *via* a Bradford assay, before being stored at -80°C.

8.5.5 Integrity of enzymes (Solution Assays of *E. coli* Hyd-1)

The steady-state rates of oxidation of hydrogen were measured by conventional solution assays by monitoring the reduction of methylene blue (MB) at 600 nm. Ocean Optics S2000 fibre optic spectrometer controlled with OOIBase32 software (Ocean Optics, Inc.) were used within an anaerobic glovebox (Belle Technologies). A stock solution of 50 μ M methylene blue (MB) was dissolved in pH 6.0 potassium phosphate buffer

(Fisher Scientific) and diluted to a working concentration of 25 μM when needed. The working solution was continuously purged with H_2 (at least for 5-10min) of which 1 mL was then transferred to a sealed cuvette containing a stirrer bar. The cuvette was then placed in the UV spectrometer and the change in absorbance monitored until stable. 1–5 μL of the enzyme (in both cases of WT and P508A variant were made to be 1–5 mg/mL, as determined by Bradford Assay)¹⁵⁰ which were exhaustively activated by H_2 for at least 16 h in the glovebox just prior to the experiment were drawn into a gas-tight Hamilton syringe and injected into the sealed cuvette. The change in absorbance (initial velocities) was converted to give results in the form of $\mu\text{mol H}_2$ oxidized per minute per mg enzyme or as the apparent turnover rate, k (s^{-1}) using the extinction coefficient for MB as $22400 \text{ cm}^{-1}\text{M}^{-1}$.¹⁷⁰

8.6 Preparation of Samples for Various Other Methods

8.6.1 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) measures heat changes that occur in a sample as it is subjected to a controlled increase or decrease in temperature: the result is the heat capacity of a molecule, in aqueous solution, plotted as a function of temperature. It can be used to measure directly the stability and unfolding of proteins, lipid membranes or nucleic acids. 400 μL each of Hyd-1 native and its P508A variant were submitted to Dr David Staunton (Dept. of Biochemistry, University of Oxford, UK) to run them in Malvern VP Capillary DSC Equipment. 5mls of Buffer C (see Appendix 9) , the buffer with which the desalting column, was conditioned, was also submitted for buffer runs to serve as controls and to condition and clean the reference and sample cells of the equipment.

8.6.2 Gas Chromatography-Mass Spectroscopy (GC-MS)

Small aliquots (0.5 mL) of the solution were taken from the electrolytic cell periodically by syringe extraction through the side rubber septum seal and the product sealed and submitted to Dr. James Wickens (CRL, University of Oxford, UK) for GC-MS headspace analysis. Calibration was established with standard solutions of PCE, TCE, and *cis*-DCE initially dissolved in ethanol and then made up to volume with 100mM TRIS and 4mM (NH₄)₂SO₄(with toluene introduced as internal standard). An Agilent 7890B model fitted with a HP5 column (He carrier gas) was used for carrying out GC-MS. The sampling vial was incubated at 60°C for 5 min before a sample of the headspace was taken by an auto-sampling needle. The GC oven was held at 40°C for 5 min after injection, the temperature was then ramped up to 290°C at a rate of 50° C per minute.

8.6.3 Ion- Selective Electrode

Pyrolytic graphite electrodes were modified to allow enough adsorbed enzyme such that probability of formation of ammonia in detectable quantities was maximized. The modification was carried out using a multi-walled carbon nanotube (MWNT)/activated 1-pyrenebutyric acid system as outlined by Krishnan *et al*¹²⁰ and described in Section 8.1. The enzyme CODH I_{Ch} (13.3 mg/ml) was used for this work. A volume of 5 µl of this enzyme was spotted onto the electrode during modification which was subsequently placed into 200 mM MES buffer (pH 7) with 20 mM hydroxylamine solution. The electrolytic solution was collected after 12 hours of poisoning the electrode at –1 V vs SCE and submitted to Dr. Ian McPherson (Tsang group, ICL, University of Oxford, UK) to use an Ion-Selective electrode and low-level Ionic Strength Adjustors (ISA) (Orion™) for the detection of ammonia. The pH-adjusting ISA was utilized to

provide a uniform background for the readings. The electrolytic solution from a control experiment without the enzyme was also submitted. Samples of 200 mM MES buffer (pH 7) with 20 mM hydroxylamine solution were also submitted to provide background readings.

8.6.4 Nuclear Magnetic Resonance (NMR)

For maximizing the chances of detectable amounts of formate, a PGE electrode was modified using a multi-walled carbon nanotube (MWNT)/activated 1-pyrenebutyric acid system as described by Krishnan *et al.*¹²⁰ 10 μ l of CODH I_{Ch} (of measured activity of 1300 μ mol min⁻¹ mg⁻¹) was applied onto the resulting electrode The electrode was then placed in a 3 mL solution of 200 mM MES (55.5% H₂O and 45.5% D₂O (99.9 % Apollo Scientific), pH 7) in the electrochemical cell and poised at -1 V vs SCE with a continuous flow of CO₂ (40 scc/min) through the cell headspace. The electrode was rotated at 400 rpm. An aliquot of 400 μ l of the solution were placed in an NMR tube and submitted to Dr. Nick Rees (CRL, University of Oxford, UK) for analysis. The NMR analysis was performed using a Bruker AVIII HD 500 model and analysed by Topspin 3.5 software.

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Appendix

Appendix 1: Non-Turnover signals

Non-turnover signals are obtained when redox centres with the enzymes respond to the sweep in voltages by yielding an oxidative peak and a reductive peak in the voltammogram. Since there is no catalysis occurring (due to absence of substrate), these peaks are called ‘non-turnover’ peaks. Their shapes are function of the total coverage of protein on the electrode and is given by

$$i = \frac{\pm n^2 F^2 v A \Gamma}{RT} \left(\frac{\exp\left(\frac{nF(E - E^{o'})}{RT}\right)}{\left(1 + \exp\left(\frac{nF(E - E^{o'})}{RT}\right)\right)^2} \right)$$

As the potential E varies as a function of time in a cyclic voltammogram, the change in surface coverage of the oxidised or reduced species with respect to time, $\frac{d\Gamma_{ox}}{dt}$ or $\frac{d\Gamma_{red}}{dt}$, is related to the change in surface coverage of the oxidised or reduced species with respect to potential, $\frac{d\Gamma_{ox}}{dE}$ or $\frac{d\Gamma_{red}}{dE}$, and can be found by multiplying them with the scan rate v .¹²²

$$\begin{aligned} \frac{d\Gamma_{ox}}{dt} &= v \frac{d\Gamma_{ox}}{dE} \\ \frac{d\Gamma_{red}}{dt} &= v \frac{d\Gamma_{red}}{dE} \end{aligned}$$

According to Nernst equation, $E = E^{o'} + \frac{RT}{nF} \ln\left(\frac{\Gamma_{ox}}{\Gamma_{red}}\right)$, where Γ_{ox} and Γ_{red} are the surface coverage of the oxidised or reduced species. Since $\Gamma = \Gamma_{ox} + \Gamma_{red}$, the surface coverage of the oxidised or reduced species, Γ_{ox} and Γ_{red} , may be given by the expression $\frac{\Gamma}{1 + \exp\left(\frac{nF(E - E^{o'})}{RT}\right)}$.

This expression may then be differentiated to yield the following equation

$$\frac{d\Gamma_{ox}}{dE} \text{ or } \frac{d\Gamma_{red}}{dE} = \frac{-\frac{nF\Gamma}{RT} \exp\left(\frac{nF(E - E^{o'})}{RT}\right)}{\left(1 + \exp\left(\frac{nF(E - E^{o'})}{RT}\right)\right)^2}$$

which can be converted into differentials with respect to time by multiplying by the scan rate. This form can then be substituted into the relationship applicable for redox centres in bound proteins:¹²² $i = -nAF \frac{d\Gamma_{ox}}{dt} = nAF \frac{d\Gamma_{red}}{dt}$ to yield

$$i = \pm \frac{n^2 F^2 A \Gamma v}{RT} \frac{\exp\left(\frac{nF(E - E^{0'})}{RT}\right)}{\left(1 + \exp\left(\frac{nF(E - E^{0'})}{RT}\right)\right)^2}$$

Appendix 2: Levich Plots

Levich plots refer to Koutecky-Levich plots which were used to determine the optimal rotation rates for the electrode during $K_M^{H_2}$ experimentations. An example is given in Figure A1.

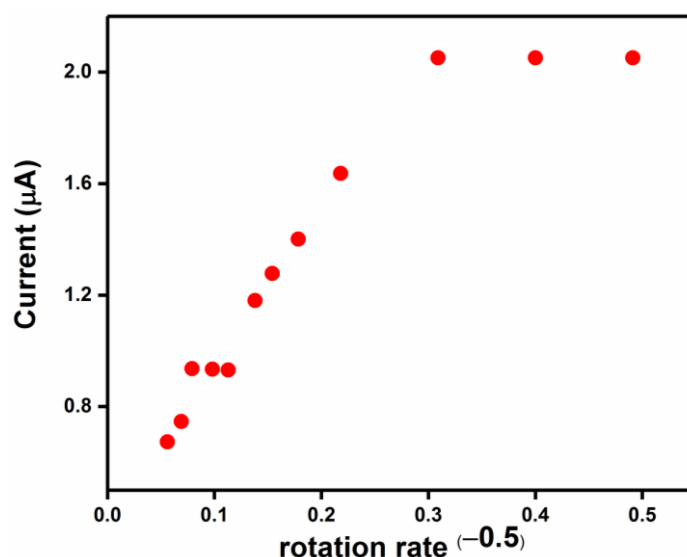


Figure A1: The results of performing Levich analysis where high rotation rates result in the levelling of the current. The experiments are carried out at the lowest substrate concentrations to ensure that the rotation rate is sufficient to ensure mass transfer is *not* rate-limiting during the experiments.

The relationship between the observed current and the rotation rate is given by the Koutecky-Levich equation:

$$\frac{i}{i_{lim}(v)} = \frac{1}{i_{lim}} + \frac{1}{0.62 * nFAD^{\frac{2}{3}}v^{-\frac{1}{6}}w^{\frac{1}{2}}C}$$

where i_{lim} is the observed current at rotation rate w and i_{max} corresponds to the maximum limiting current at an infinite rotation rate. The rotation rate required to ensure that the current observed during a Protein Film electrochemistry (PFE) experiment is independent of mass-transport, corresponding to i_{max} , can therefore be determined from a plot of i_{lim} against $\frac{1}{w^{\frac{1}{2}}}$ known as a Koutecky-Levich plot.

Appendix 3: Accounting for Film Loss

Film loss occurs in PFE experiments and is observed as a gradual decrease in current over the course of the experiment even when the experimental conditions are constant, *i.e.*, unchanging potential, pH, temperature, *etc* are maintained. This may be due to enzyme molecules dissociating from the electrodes or from re-orientating on the electrode surface and causing them to lose electronic contact with the electrode or from enzyme molecules denaturing on the electrode surface. In order to account for it, it is necessary to define a baseline condition to which the experimental conditions are returned to periodically during the course of the experiment (*e.g.* 100% H_2 during $K_M^{H_2}$ experiments).

This data is then used to plot an exponential decay function of the form $y = A_1 e^{\left(\frac{-x}{t_1}\right)} + y_0$ (Figure A2) to generate a correction constant per current value, where Z is the correction constant, t is time and the other constants are the values obtained from the exponential decay function.

$$Z = A_1 e^{\left(\frac{-t}{t_1}\right)} + y_0$$

The correction constant is then divided by the current at the start of the experiment ($t = 0$, $i_{initial}$) to obtain a correction ratio, C : $C = \frac{Z}{i_{initial}}$ such that the current corrected for film loss

(i_{corr}) is given by $i_{corr} = \frac{C}{i_t}$.

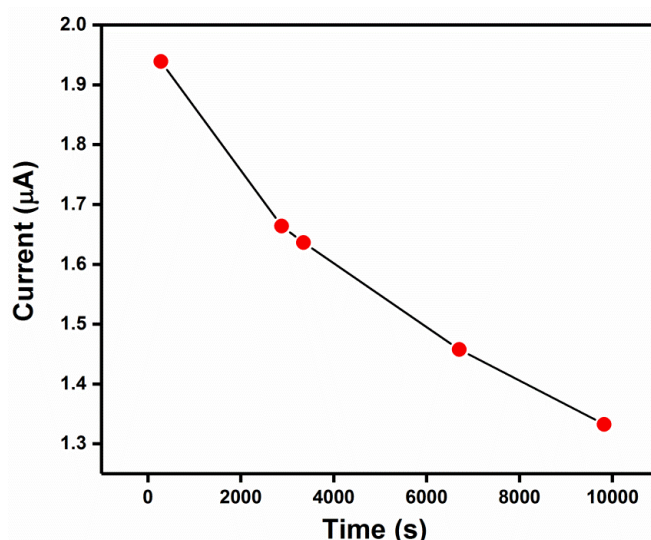


Figure A2: In order to correct for film loss, it is necessary to define a baseline condition to which the experimental conditions are returned to periodically during the course of the experiment (*e.g.* 100% H_2 during $K_M^{H_2}$ experiments). The data points are then used to plot an exponential decay function of the form $y = A_1 e^{\left(\frac{-x}{t_1}\right)} + y_0$.

Appendix 4: Hanes-Woolf analysis for calculating Michaelis-Menten constant

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) which then dissociates to release back free enzyme and product (P), then for the reaction



where the assumption is made that the rate of formation of the ES complex is rapid relative to the rate of dissociation, a steady state approximation may be applied to give

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

By rearrangement;

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

Then if $K_M = (k_{-1} + k_{cat})/k_1$

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

If we now consider the total amount of enzyme present, $[E]_0$, as

$$[E]_0 = [E] + [ES]$$

then rearrangement and substitution gives the value of $[ES]$ as

$$[ES] = \frac{[E]_0[S]}{K_M + [S]}$$

The rate of the reaction, v , is given by;

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

and the maximum rate of the reaction, v_{max} , is obtained when all the enzyme molecules are saturated with substrate so that $[ES] = [E]_0$.

Then

$$v_{max} = k_{cat} [E]_0$$

which can be substituted to give:

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

This is the Michaelis-Menten equation and from this equation a Hanes-Woolf analysis can be carried out. The above equation can be rearranged to give

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

from which a plot of $\frac{[S]}{v}$ vs $[S]$ where $[S]$ is concentration of substrate, S, in mol dm^{-3} gives a

slope of $\frac{1}{v_{max}}$, a value which is then utilized to give K_M from the y-intercept. In lieu of v

v_{max} , the values of the current (i) and (i_{max}) are measured and recorded since it is directly proportional to k_{cat} as given by Various enzymes were demonstrated to be excellent *electrocatalysts*, catalysts which enhance electrochemical reactions,^{29, 31} by the PFE technique

and many of them operate with minimal overpotentials on pyrolytic graphite edge (PGE) electrodes.^{29, 32} in the main text

$$i_{cat} = A\Gamma Fnk_{cat}$$

where A= area of the electrode surface, Γ = electroactive coverage by enzyme, F= Faraday's constant and n= number of electrons involved in the reaction. If S is gaseous, then the % composition of S in the gas flow in the headspace is converted to [S] using Henry's law, given in Equation 21 from the main text (reproduced here):

$$c_g = k_H p_g$$

where c_g is the concentration of dissolved gas, p_g is its partial pressure and k_H is Henry's Law constant.

Appendix 5: Preparation of RbCl Competent Cells

The RbCl method of making competent cells is describe by Hanahan *et al.*¹⁷¹ *E. coli* cells to be made competent are grown on LB plates at 37 °C for 16 hours. A single colony was then used to inoculate 5 mL LB which was grown overnight at 37 °C and 200 rpm. 100 μ L aliquot of it was then used to inoculate 50 mL LB and the culture grown until an OD₆₀₀ of 0.4 was reached. The culture was then placed on ice before centrifugation at 3000 rpm for 10 minutes (Beckmann Centrifuge GS-6R) at 4 °C before Buffer TFB-I (Appendix 9) was used to resuspend the cells. The resuspended was placed on ice for 15 minutes before further centrifugation at 3000 rpm for 10 minutes. 2 ml of Buffer TFB-II was then used to resuspend the cell pellet which was subsequently placed on ice for 15 minutes before being divided into 100 μ L aliquots to be “flash frozen” in liquid nitrogen and stored at –80 °C.

Appendix 6: Transformation into Competent Cells

Plasmid DNA (~1 μ L) was added to 1ml of competent cells and put on ice for 30 minutes. A heat shock step was then performed at 42 °C for 40 seconds before the cells were incubated on ice for a further 2 minutes and 1 mL LB subsequently added. This was incubated at 30 °C for 4 hours, after which the cells were pelleted *via* centrifugation at 6500 rpm for 5 minutes (Eppendorf Centrifuge 5415C). The cell pellets were then suspended using a small amount of supernatant and plated.

Appendix 7: Protocol for Hyd-1 prep for PFE and Crystallization

This protocol is reproduced from FAA Group records. Composition of the various buffers utilized during isolation of enzyme from a culture of *E. coli* are given in Appendix 9.

Inoculation day:

- Media: autoclave 10x(150g of high salt LB(melford L1704) in 6L bottle, add 5L of DI water), 10x1L of DI water and 600ml of 50% glycerol.
- Starters: 5 ml LB in falcon tube, 37 °C, 6 hours.
- Sodium fumarate solution: dissolve 240g of sodium fumarate in 1200 ml of DI water; bring volume up to 1500 ml, filter- sterilize.
- Inoculation: add 60 ml of 50% glycerol autoclaved solution to 5L of 37 °C media, add 150 ml of sodium fumarate solution, add 5ml starter, top up with autoclaved DI water.
- Growing: 37 °C stationary incubator for 17 hours.

Harvest day:

- Spin: 5000 rpm for 12 minutes, 4 °C. Take bottles out of the incubator one by one.
- Make re-suspension buffer: add DNase and lysozyme to 210 ml of buffer 1.
- Add 35 ml of re-suspension buffer to each 1L centrifuge tube, re-suspend in room temperature shaker for about 30 minutes (200 rpm).
- Freeze: 50 ml falcon tubes, -80 °C.

French press day:

- Thaw cells in room temperature water bath/shaker.
- Add 1mM DTT.

- French press 2 times, 20 kPSI.
- 1st spin: 30 minutes, 4 °C, 3600 rpm, discard supernatant.
- Extra spin for unlyzed cells at 25 kpsi.
- 2nd spin: 60 min, 45000 rpm, 4 °C. Membrane homogenizing: add some buffer2 (add DTT) to each tube, re-suspend pellet with plastic pipette and combine, homogenize using cell homogenizer. Bring volume up to 270 ml. Leave in the fridge overnight.

Column day:

- Solubilising membranes: add 30 ml of 30% Triton X100 solution to 270 ml of membrane suspension and put on the stirring plate for 40 min in cold room.
- Add DTT to buffers A (250 ml) and B(150 ml).
- Spin: 45000 rpm, 60 minutes, 4 °C.
- Wash column with buffer A (3 ml/min, 50 ml, AKTA).
- Add imidazole (40 mM) and DTT (1 mM) to supernatant.
- Load supernatant onto the HP HisTrap 5 ml column at 2 ml/min.
- Wash column with 200ml(40CV) of buffer A.
- Run the gradient: 1.5 ml/min, 100% buffer B in 67 min (20 CV); 1 ml fractions, end timer 100 ml).
- Run a gel.
- Collect fractions in 20 ml concentrator.
- Concentrate using bench top centrifuge (3600 rpm, 4 °C) to 1 ml.
- Run desalting column (conditioned with buffer C), collect, freeze.

If the prep is to be crystallized then instead of running through a desalting column, the following steps are taken:

- Transfer sample to dialysis tubing (MWCO 3,500).
- Leave stirring overnight in the cold room in 3L of dialysis buffer.

For crystallization

Size exclusion column/ hydroxyapatite column day:

- Transfer to 1L of fresh dialysis buffer.
- Take it to Harwell on ice.
- Transfer samples to 2L of fresh dialysis buffer, stirring plate for 2 hours, cold room.
- Start conditioning size exclusion column (Superdex tm 200 10/300 GL, GE 17-5175-01) with dialysis buffer(1L).Flow rate 0.5 ml/min; 30-60 ml.
- Load sample onto the column with a syringe. Elute using same dialysis buffer.
- Run a gel

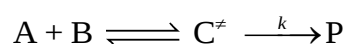
- Pool 3rd peak fractions together.
- Equilibrate hydroxyapatite column with 50 ml of buffer HT1 (1 ml/min, peristaltic pump).
- Load, 1 ml/min.
- Transfer to AKTA.
- Wash the column with buffer HT1 (5 cv), 1.5 ml/min. Important!
- Gradient (100% HT2 in 10CV, 1.5 ml/min).
- Run a gel.
- Collect 1st peak fractions, concentrate (3000rpm) down to 1.5-1.7 ml.
- Collect 2nd peak fractions, concentrate down to 1.5-1.7 ml.
- Transfer samples to dialysis tubing, leave overnight in 2L of dialysis buffer stirring at 4 °C.

Crystallization tray day:

- Transfer samples to 2L of dialysis buffer, stirring plate for 2 hours.
- Bradford test.
- Dilute or concentrate samples in order to have 5 mg/ml concentration.
- Set up crystallization trays: Sitting-drop vapour diffusion method, mixing 1.5 uL protein solution with 1.5 uL 100mM Bis-Tris pH 5.9, 21-23% PEG 3350, 200 mM LiSO₄, 150 mM NaCl followed by incubation at 293K.

Appendix 8: The Eyring Equation

The Eyring equation is based on Transition State Theory in which reaction between A and B occurs *via* the formation of a state which then subsequently breaks down to give the product,



The rate constant, k , can then be given by

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

which is the Eyring Equation, in which k is the rate constant for the reaction, k_B is the Boltzmann's constant, T is the temperature in Kelvin, h is the Plank's constant, R is the gas constant. Applying natural logarithmic transformation gives

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

A plot of $\ln(k/T)$ vs. $(1/T)$ gives a straight line from which the gradient reading of $-\frac{\Delta H^\ddagger}{R}$ can be used to give the activation enthalpy, $\Delta H^\ddagger$, and the y-intercept of $\ln\left(\frac{k_B}{h}\right) + -\frac{\Delta S^\ddagger}{R}$ can be used to calculate the entropy change, $\Delta S^\ddagger$.

The relationship between rate constant (k) and catalytic current (i) is given below:

$$i = A\Gamma F n k$$

in PFE experiments in which n is the number of electrons involved in the reaction, F is the faraday constant, A is the electrode area and Γ is the electroactive coverage. The values of i from the experiments are used to substitute k in utilizing the Eyring equation but the value of Γ remaining unknown, it is a challenge to calculate $\Delta S^\ddagger$.

Appendix 9: Buffers utilized in molecular biology work

Table A 1: Variation in canopy residues across the [NiFe]-hydrogenase subgroups.

BUFFER	PURPOSE	COMPONENTS	PH
TFB I	Used to make <i>E.coli</i> chemically competent for transformation	3 mM hexamine cobalt chloride, 15% glycerol, 50 mM MnCl ₂ , 10 mM CaCl ₂ , 100 mM RbCl, 30 mM KOAc	5.8
TFB II	Used to make <i>E. coli</i> chemically competent for transformation	10 mM MOPS, 75 mM CaCl ₂ , 10 mM RbCl, 15% glycerol	6.5
TAE	Running buffer for agarose gel electrophoresis	1mM EDTA, 40 mM Tris, 40 mM glacial acetic acid	8.3
Buffer 1	Resuspension of pelleted harvested cells	100 mM Tris, 1 mM EDTA, 50 mM NaCl	7.5
Buffer 2	Solubilisation of cell membranes	100 mM Tris, 50 mM NaCl	7.5
Buffer A	Washing of Histrap column	20 mM Tris, 100 mM NaCl, 60 mM imidazole, 1 mM DTT, 0.02% (w/v) DDM	7.2
Buffer B	Protein elution from Histrap column	20 mM Tris, 100 mM NaCl, 500 mM imidazole, 1 mM DTT, 0.02% (w/v) DDM	7.2
Buffer C	Desalting and storage of proteins at -80 °C	20 mM Tris, 150 mM NaCl, 1 mM DTT, 0.02% (w/v) DDM, 10% (v/v) glycerol	7.2
Dialysis buffer		20 mM Tris-HCl, 150 mM NaCl, 1mM DTT, 0.02% (w/v) DDM	7.2
HT1 buffer		10 mM K ₂ HPO ₄ /KH ₂ PO ₄ , 150mM NaCl, 1 mM DTT, 0.02% (w/v) DDM	7.2
HT2 buffer		500 mM K ₂ HPO ₄ /KH ₂ PO ₄ , 150mM NaCl, 1 mM DTT, 0.02% (w/v) DDM	7.2

Appendix10: Variation in canopy residues across [NiFe]-hydrogenase subgroups

Table A 2: Variation in P508 residues across the [NiFe]-hydrogenase subgroups. A detailed analysis of the possible amino acids in positions equivalent to P508 across all different groups of [NiFe] –hydrogenases based on an extended sequence alignment was carried out by Dr. Rhiannon Evans (for a study of variation and effect of canopy residues by Brooke *et al*^{126a}). The (meta)genomic analysis from Greening *et al*⁵⁵ was used as a sequence database and includes sequences of putative hydrogenases classified according to metal binding motifs and genome/domain organisation.^{54c} Adapted from Brooke *et al*.^{126a}

[NiFe(Se)] groups aligned against Group 1 to identify Hyd-1 canopy equivalent residues	Group 1 – Membrane bound H₂ uptake: • 1A = Ancestral – includes NiFeSe (sulfate, organohalide, methanogenic and heterodisulfide respiration) • 1B = Prototypical (sulfate, fumarate, nitrate respiration) • 1C = Hyb type (includes Hyd2) – fumarate reduction, possibly bidirectional • 1D = O₂ tolerant (includes Hyd-1) – aerobic respiration and O₂ tolerant anaerobic respiration • 1E = Isp type – sulfur respiration, possibly bidirectional • 1F = unresolved – may be linked to reduction of reactive oxygen species • 1G = unresolved – may be linked to sulfur respiration • 1H/5 = Tropospheric H₂ scavenging in aerobic respiration/starvation	Group 2 – Cytosolic H₂ uptake: • 2A = Cyanobacteria type – aerobic respiration, H₂ recycling (from nitrogenase, fermentation) • 2B = HK linked – Senses H₂ and activates two-component cascade controlling H₂ase expression • 2C = DGC linked (putative) – predicted to sense H₂ and induce cyclic di-GMP production • 2D = Aquificae type – unknown. May generate reductant for C fixation	Group 3 – cytosolic bidirectional: • 3A = F₄₂₀ coupled – Directly couples oxidation of H₂ to reduction of F₄₂₀ during methanogenesis. Reverse reaction may also occur. Includes [NiFeSe] variants • 3B: NADP coupled - Directly couples oxidation of NADPH to evolution of H₂. May be reversible. Some complexes are proposed to have sulfhydrogenase activity • 3C: HDR linked – Bifurcates electrons from H₂ to heterodisulfide and ferredoxin in methanogens without cytochromes • 3D: NAD coupled – Directly interconverts electrons between H₂ and NAD depending on redox state	Group 4 – Membrane bound H₂ evolving: • 4A = FHL type – Couples oxidation of formate to fermentative H₂. Hyf-type complexes may translocate H⁺ via antiporter modules • 4B = Mrp linked – Couples oxidation of HCO₂ or CO to proton reduction. Generates Na-motive force via Mrp antiporter modules • 4C = CODH linked – Forms complex with CODH to anaerobically respire CO using H⁺ as terminal e⁻ acceptors. • 4D = Eha/Ehb type – Multimeric, H₂ oxidation/ferredoxin reduction for anaplerotic (Eha) and anabolic (Ehb) purposes. H⁺/Na⁺ driven • Group 4e: Ech type – Couples ferredoxin oxidation to H₂ evolution. This process is physiologically reversible via H⁺/Na⁺ translocation • Group 4f: Ehf type (putative) – Unknown. May couple oxidation of a one-carbon compound to proton reduction concurrent with proton translocation. Related to Ehr complexes.
Number of sequences identified	781	207	549	461
P508 conservation	DEPENDS ON SUBGROUP: ○ 1A = P A S M G T I V ○ 1B = P A T ○ 1C = P S ○ 1D = P ○ 1E = P ○ 1F = P A S ○ 1G = P G ○ 1H/5 = V	DEPENDS ON SUBGROUP: ○ 2A = A I S M ○ 2B = A ○ 2C = A P V ○ 2D = A S T C P	DEPENDS ON SUBGROUP: ○ 3A = P A ○ 3B = P A ○ 3C = P N A T S ○ 3D = P	DEPENDS ON SUBGROUP: ○ 4A = P ○ 4B = P H ○ 4C = P ○ 4D = P Q H ○ 4E = P S ○ 4F = P A

