

Inhibition of [NiFe]-hydrogenases with π -acid ligands:
electrochemical and *in situ* infrared spectroelectrochemical studies



Min-Wen Chung

St John's College

Trinity Term 2015

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

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Hydrogenases are of great interest because they utilise abundant and inexpensive metals, Fe or Ni-Fe, in their active sites to catalyse hydrogen cycling – highly relevant to enzyme-based or bio-inspired technologies. The Fe in the active site is in a biologically unusual ligand environment coordinated by CO and CN⁻. These small molecule ligands have intense infrared absorption features and serve as good probes to monitor the coordination state and electronic environment of the active site. Infrared spectroscopy has therefore become a standard approach for characterising hydrogenases isolated from different organisms, but to date only few spectroscopic results have been correlated with states of the active site generated during catalysis. A new approach, PFIRE, which couples direct electrochemical control of hydrogenase, immobilised on a carbon particle electrode, and FTIR spectroscopy together is exploited in this Thesis to address this issue. This Thesis develops a surfactant-modified particle electrode to have a high loading of *E. coli* Hyd-2 (a standard [NiFe]-hydrogenase) for use in PFIRE for comparison with the O₂-tolerant membrane-bound hydrogenase, *E. coli* Hyd-1 during catalytic turnover. A systematic study of how *E. coli* Hyd-1 and *E. coli* Hyd-2 respond to other π -acid ligands is also reported in this Thesis. Cyanide was found to have unusual reactions with Hyd-1 and Hyd-2; it can promote the formation of the oxidised inactive state, Ni-B. Isocyanides are shown for the first time as inhibitors of [NiFe]-hydrogenases.

The use of bulky isocyanides as probes in this Thesis reveals that the catalytic pocket of Hyd-1 is more spacious than previously thought, as even *n*-butyl isocyanide with a long tail is capable of getting in and out to react with the active site reversibly. Overall this Thesis provides a view of how π -acid ligands react with a prototype O₂-sensitive and O₂-tolerant [NiFe]-hydrogenase.

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Abbreviations

ATP	adenosine triphosphate
FTIR	Fourier transform infrared
EPR	electron paramagnetic resonance
PFE	protein film electrochemistry
DFT	density functional theory
ATR	attenuated total reflectance
PFIRE	protein film infrared electrochemistry
NMR	nuclear magnetic resonance
CE	counter electrode
WE	working electrode
RE	reference electrode
PGE	pyrolytic graphite 'edge'
SCE	saturated calomel electrode
SHE	standard hydrogen electrode
Hyd-1	[NiFe]-hydrogenase 1 from <i>Escherichia coli</i>
Hyd-2	[NiFe]-hydrogenase 2 from <i>Escherichia coli</i>
MBH	membrane bound hydrogenase
SH	soluble hydrogenase
RH	regulatory hydrogenase
DvMF	<i>Desulfovibrio vulgaris</i> Miyazaki F
<i>D. fructosovorans</i>	<i>Desulfovibrio fructosovorans</i>
<i>A. vinosum</i>	<i>Allochromatium vinosum</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>D. gigas</i>	<i>Desulfovibrio gigas</i>
Da	Dalton
V	volt(s)
mV	millivolt(s)
s	second(s)
A	ampere(s)

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Chapter 1: Introduction

1.1 Hydrogenases

1.1.1 Overview

Hydrogenases are metalloproteins from microorganisms that catalyse an interconversion between H_2 and protons, as shown in equation [1-1], at high turnover frequencies.¹



The use of abundant and inexpensive metals, Fe or NiFe, in their active sites for dihydrogen activation is of great interest because dihydrogen is regarded as a sustainable fuel with prospects, but presently precious metals are used for its activation. A detailed understanding of how molecular hydrogen is activated in the enzymes provides many important design principles for biomimetic catalysts,²⁻⁵ which could replace noble metals, such as platinum for application in energy cycling in the future.

Hydrogen: an energy source in the microbial world

In nature, H_2 serves as an energy source for a range of microorganisms. It is activated by hydrogenases, and in some microbes is involved in the respiratory process coupled with different electron acceptors, including O_2 (aerobic respiration), nitrate, fumarate, Fe^{3+} , or sulphate for anaerobic respiration. Given that the hydrogenases from *Desulfovibrio* species have been widely

studied, Figure 1-1 depicts the simple chemiosmotic mechanism of energy conservation by H_2 cycling of sulphate-reducing bacteria *Desulfovibrio*.^{6,7} The oxidation of H_2 catalysed by periplasmic hydrogenases generates a proton gradient across the cytoplasmic membrane, and the electrons are transferred via electron transfer proteins to the electron acceptor, sulphate, located in the cytoplasm. The energy generated by the oxidation of H_2 coupled to SO_4^{2-} reduction supports ATP formation. Dihydrogen can also be produced from organic substrates in the cytoplasm, and can then diffuse across the cytoplasmic membrane to be oxidised in the periplasm.

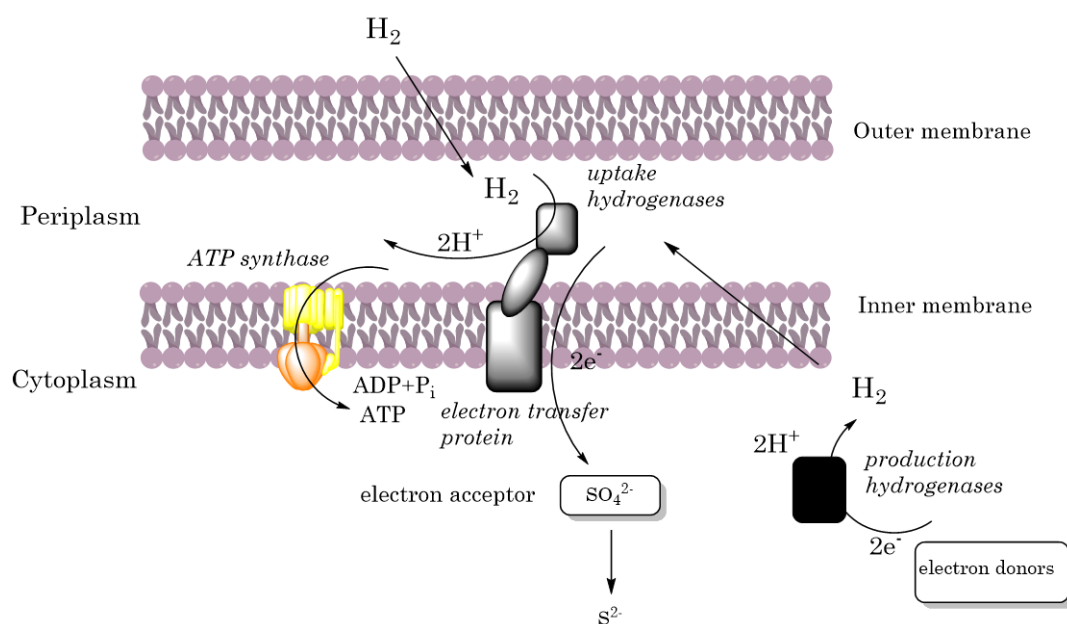


Figure 1-1. A simple chemiosmotic mechanism of hydrogen cycling for energy conservation inside sulphate-reducing bacteria *Desulfovibrio*.^{6,7}

Hydrogenases are also able to balance the redox potential of the cell via catalysing hydrogen cycling, i.e. excess electrons can be removed by proton reduction, or electrons are provided by cleavage of molecular hydrogen. In this

respect, hydrogenases either are physiologically tuned for H_2 production or H_2 uptake (but can offer work in both directions to some extent), depending on their location in the cell.⁸

Hydrogen flow among microbial communities

The reducing power of H_2 plays an important role in cell metabolism for anaerobic microorganisms. Therefore, H_2 formed geologically and biologically is rapidly consumed by different microbial communities that encounter it, with very little being released into the atmosphere. Figure 1-2 illustrates H_2 cycling through different zones in an aquatic microbial ecosystem, according to a vertical redox potential of microbial substrate reduction coupled to H_2 oxidation defined by Conrad.⁹ In anaerobic regions, fermentative bacteria produce H_2 in submerged sediments, and the H_2 is consumed by methanogens, reducing CO_2 to CH_4 . Then, some H_2 diffuses upward further to the sulphate, Fe^{3+} and nitrate reduction zones.

In upland soils, the conditions are aerobic due to contact with the air and O_2 produced by photosynthetic bacteria. However, only trace levels of H_2 reach this region at the surface, and the other source of H_2 here is from the byproduct of ammonia formation catalysed by nitrogenases in soil bacteria and N_2 -fixing root nodules. In this region, H_2 is exploited by aerobic bacteria to reduce O_2 for energy generation.

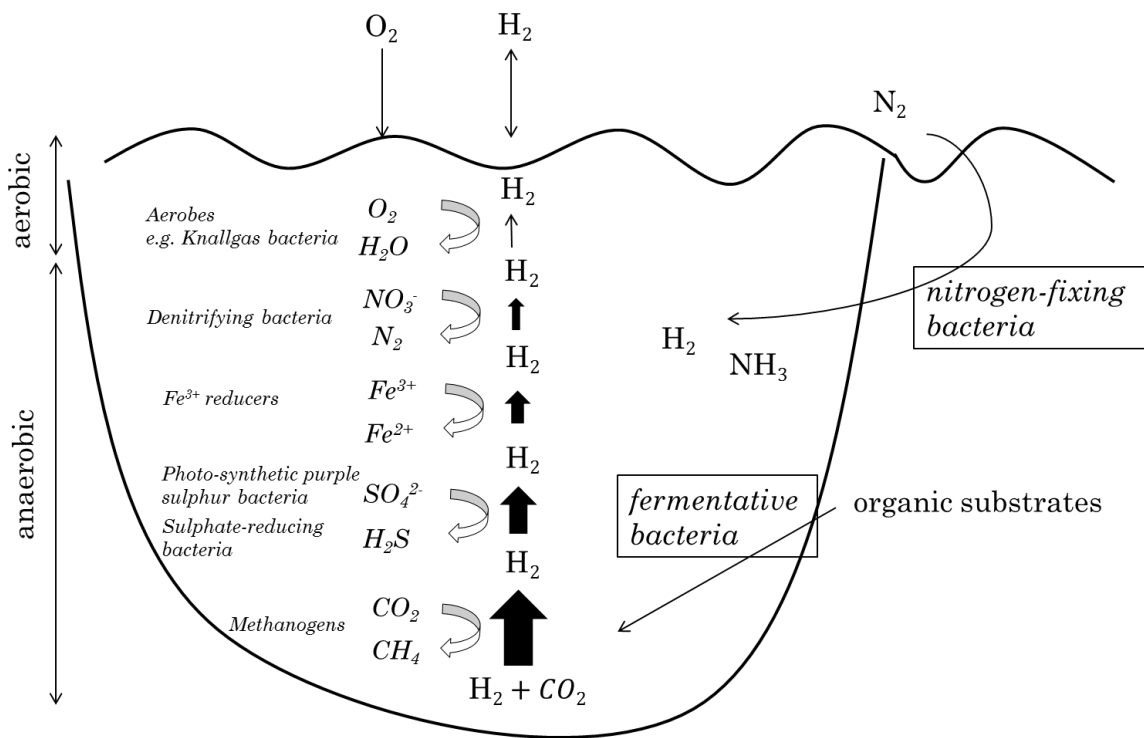


Figure 1-2. A schematic representation of the flow of H₂ in an aquatic microbial ecosystem. The diagram is adapted and reprinted with permission from ref 9 Copyright © 1996, American Society for Microbiology

1.1.2 Types of hydrogenases

According to the metal composition in the active sites, hydrogenases can be classified into three phylogenetically unrelated groups, [FeFe]-, [NiFe]-, and [Fe]-hydrogenases, as shown in Figure 1-3.^{1,10}

[FeFe]-hydrogenases

[FeFe]-hydrogenases are found in anaerobic bacteria and some unicellular eukaryotes, and generally have a physiological role in H₂ production.^{11,12} The catalytic site, known as the H-cluster, is composed of a ferredoxin-like [4Fe4S] cluster, connected to a binuclear [FeFe] centre via a cysteine sulphur bridge (Figure 1-3, panel a). Each iron of the binuclear centre is coordinated by CO and CN⁻ ligands. A five-atom dithiolate ligand with a bridgehead atom of N links the two irons together. The distal iron (with respect to the position of the [4Fe4S] cluster) is proposed to be involved in H₂ binding, as it has an open coordination site, to which exogenous CO can bind as an inhibitor. Also, the distal iron is redox active during the catalysis.

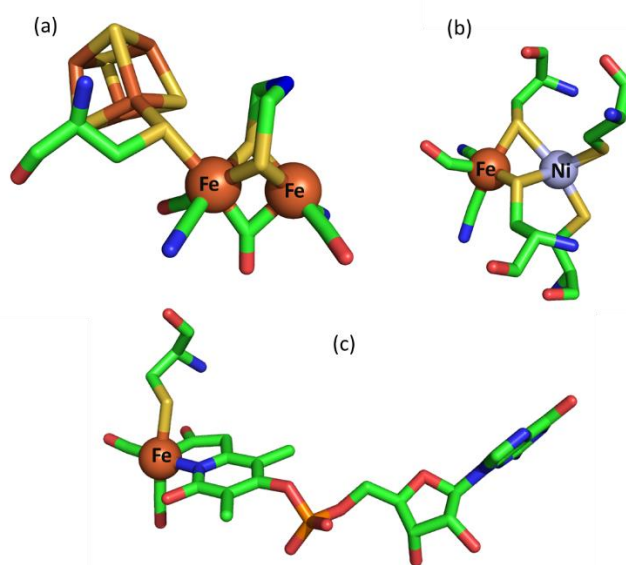


Figure 1-3. Active-site structures of the three phylogenetically unrelated hydrogenases: (a) [FeFe]-hydrogenase; (b) [NiFe]-hydrogenase; and (c) [Fe]-hydrogenase. The metal centres are shown as spheres, and all atoms are represented in standard colours.

[NiFe]-hydrogenases

[NiFe]-hydrogenases are present in bacteria and archaea,^{11,12} and the most well-studied examples are composed of two moieties, a large subunit (~ 60 kDa), and a small subunit (~ 40 kDa). The active site is linked to the protein in the large subunit via four cysteine sulphurs, two of which bridge the metals, and the remaining two are terminal ligands to the nickel. The iron is further coordinated by one CO and two CN⁻ ligands (Figure 1-3, panel b). In contrast to [FeFe]-hydrogenases, the physiological function of [NiFe]-hydrogenases is to catalyse dihydrogen oxidation in most cases, with the catalytic activity at least ten times less than that of [FeFe]-hydrogenases.¹³ Also, [NiFe]-hydrogenases

are generally less O₂ sensitive than [FeFe]-hydrogenases; the attack of O₂ on a [NiFe]-hydrogenase results in inactivation but generally does not destroy the enzymes permanently as happens for [FeFe]-hydrogenases.

This thesis focuses on studies of [NiFe]-hydrogenases, and hence the architecture of the whole [NiFe]-hydrogenase will be discussed in more detail in section 1.3.

[Fe]-hydrogenases

[Fe]-hydrogenases, previously called iron-sulphur cluster-free hydrogenases or H₂-forming methylene-tetrahydromethanopterin (methylene-H₄MPT) dehydrogenases (Hmd), are only found in methanogens.¹⁴ They catalyse the reversible reduction of methenyl-tetrahydromethanopterin (methenyl-H₄MPT⁺) with H₂ to methylene-H₄MPT and H⁺ (Figure 1-4), which is an intermediary step in CO₂ reduction to methane.¹⁴ The active site contains a mononuclear iron centre, which is coordinated square-pyramidally by two CO ligands, a cysteine sulphur from the protein, and a guanylylpyridinol cofactor through an sp²-hybridised nitrogen and a formyl carbon atom (Figure 1-3, panel c).¹⁵ The sixth position appears to be occupied by a solvent molecule and is proposed to be an open site for H₂ binding.

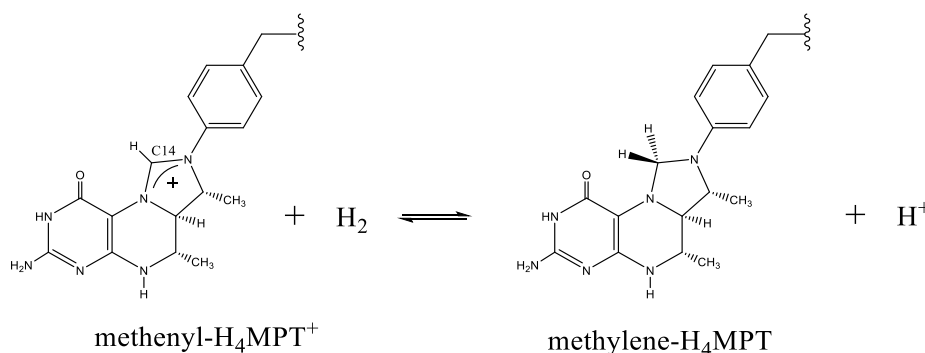


Figure 1-4. Reaction catalysed by [Fe]-hydrogenases. The carbocation character of methenyl C14 in methenyl- H_4MPT^+ makes it a good hydride acceptor to adopt hydride from heterolytic cleavage of H_2 by the enzyme.

[NiFeSe]-hydrogenases

[NiFeSe]-hydrogenases are part of [NiFe]-hydrogenases by sharing a similar pattern in an overall structural folding. In [NiFeSe]-hydrogenases, one of the terminal cysteine residues coordinating to the nickel at the active site in standard [NiFe]-hydrogenases is replaced by a selenocysteine (SeCys), but with the coordination features of the iron remaining the same. The other structural difference lies in the FeS clusters. The medial FeS cluster in [NiFeSe]-hydrogenases is a $[4\text{Fe}4\text{S}]$ cluster held by four cysteine residues to the protein, whereas it is a $[3\text{Fe}4\text{S}]$ cluster in standard [NiFe]-hydrogenases.⁸ In contrast to standard [NiFe]-hydrogenases, [NiFeSe]-hydrogenases are more active in catalysing H_2 production, even in the presence of 1% O_2 ,¹⁶ and are less inhibited by the product, H_2 . Thus, they are considered promising for technological applications.

Although these three types of hydrogenases have evolved independently, they share unique feature: the presence of low-spin iron coordinated by at least one endogenous CO ligand, which appears to play an important role in dihydrogen activation.

1.1.3 Applications of hydrogenases

Enzyme fuel cells

A fuel cell is a device that converts chemical energy into electricity. Unlike a battery, a fuel cell requires a continuous supply of oxidants and reductants, i.e. the fuel, to sustain the chemical reaction. Fuel cells have advantages over traditional fossil combustion engines; they operate at higher efficiencies, and in the case of H_2/O_2 fuel cells, the chemical reaction only produces H_2O without releasing any greenhouse gases. Figure 1-5 depicts a simple scheme of a H_2/O_2 fuel cell. H_2 is oxidised at the anode by a catalyst, and the released electrons are mediated through an external circuit to the cathode, where the O_2 is reduced to form H_2O . A membrane permeable to protons may be incorporated into the device for separation of both electrode compartments.

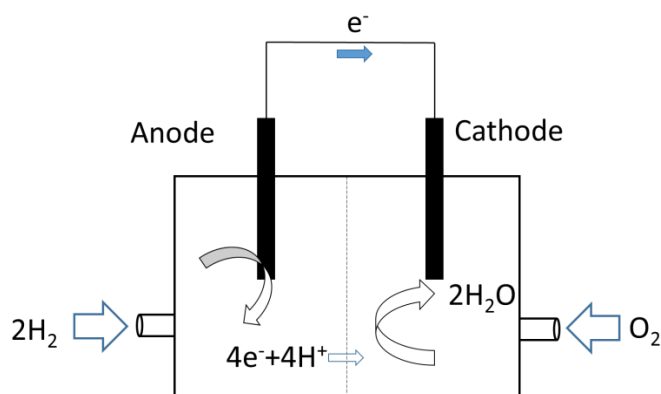


Figure 1-5. A simple schematic representation of a H_2/O_2 fuel cell.

In an enzyme fuel cell, enzymes are exploited as electrocatalysts for energy conversion. Hydrogenases use earth abundant metals (Fe and Ni) in their active site for H_2 activation, and the turnover frequency of this catalysis is

comparable to Pt per active site,¹⁷ making hydrogenases serve as a promising alternative to precious metal based catalysts used in the traditional fuel cell device. One particular advantage of enzyme fuel cells using hydrogenases is that they are not poisoned by H₂S and CO, which are typical contaminants of H₂ from steam reforming. Also, the high selectivity of enzymes in substrates could simplify the whole device structure, as a membrane for separation of anode and cathode compartments is not necessary.^{8,18}

H₂-driven heterogeneous catalyst system

A heterogeneous catalyst system can be constructed by electrically coupling hydrogenases to other enzymes, and a cheap and clean reducing agent, H₂, is used to trigger this system for valuable chemical production. Vincent *et al.* demonstrated this concept by co-immobilising a [NiFe]-hydrogenase and a nitrate reductase or a fumarate reductase on graphite particles to produce nitrite and succinate,¹⁹ which opens up possibilities of practical applications in novel biosynthesis by other redox-enzyme systems. Figure 1-6 shows a cartoon representation of this heterogeneous catalyst system.

On the basis of this concept, Reeve *et al.* designed a modular system for regeneration of adenine dinucleotide (NAD) cofactors.²⁰ NAD cofactors can transfer reducing agents in the form of a hydride, which is utilised by a range of oxidoreductases, however these expensive cofactors limit industrial biocatalysis applications. Reeve *et al.* separated NAD⁺/NADH (HoxFU) and the H⁺/H₂ (HoxHY) moieties from *Ralstonia eutropha* SH, and recombined the

HoxFU on graphite particles with *Escherichia coli* hydrogenase 2 (Hyd-2), which is a good bidirectional hydrogenase and operates at negligible overpotential. Due to the use of a more robust and active hydrogenase, this modular system can efficiently regenerate the NADH consumed by a NADH dependent enzyme during catalysis. This approach provides a promising alternative to existing cofactor recycling strategies in the field of biosynthesis.

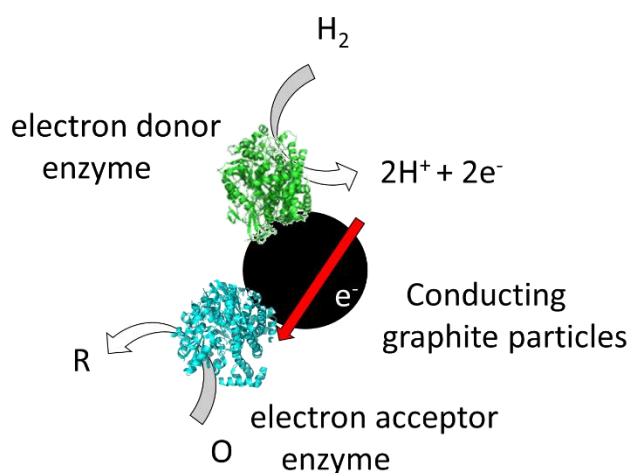


Figure 1-6. A cartoon representation of a heterogeneous catalyst system constructed on graphite particles for biosynthesis.

Photoelectrochemical H_2 production

H_2 is often referred to as an energy vector by chemists and technologists, as the energy from renewable source, e.g. solar energy, could be stored in the form of H_2 produced via electrolysis of water. And this chemically stored energy can be converted into electricity in a fuel cell efficiently. Hence, seeking renewable sources of H_2 is essential to future H_2 economy. Hambourger *et al.* reported the first example of light-driven H_2 production device in 2008 (a scheme of the device is shown in Figure 1-7).²¹ The photocurrent, generated on a porphyrin-sensitised TiO_2 particle electrode, is transferred into a carbon felt cathode via external circuit, where *Clostridium acetobutylicum* [FeFe]-hydrogenase HydA was immobilised to produce H_2 . NADH was used as a sacrificial electron donor in this system. The reported H_2 production rate was comparable to a Pt catalyst.

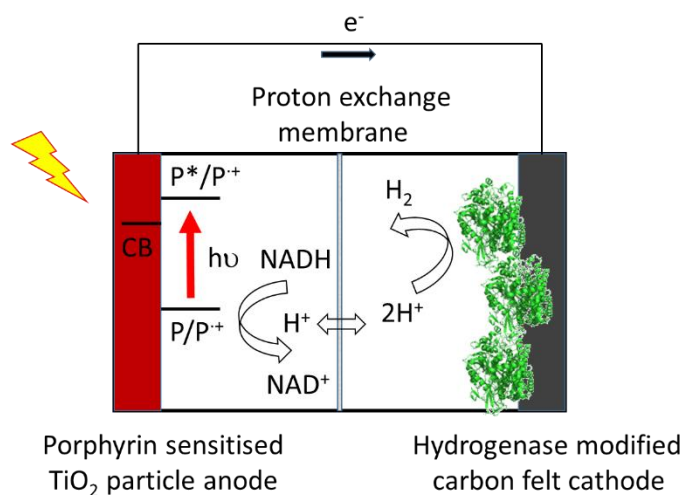


Figure 1-7. A schematic representation of the first photoelectrochemical cell for H_2 production by Hambourger *et al.*²¹

Inspiration for biomimetic chemistry

Hydrogenase model compounds

Chemists started working on biomimetic compounds of hydrogenases quickly after the crystal structures were solved, aiming at developing efficient and economic catalysts as an alternative to catalysts with precious metal contents. Design criteria inspired by nature for synthesis of efficient catalysts are: (i) the presence of an open coordination site at the metal; (ii) availability of a proton transfer base, (iii) energetic matching of the proton acceptor ability (from the base nearby) and the hydride acceptor ability (from the metal core).⁸ The first [NiFe]-catalyst showing the bidirectional behaviour observed for the native enzyme was reported by Ogo *et al.* (Figure 1-8, panel a),³ with three triethylphosphite ligands coordinating to the Fe, although the turnover number of this complex is still low and a large overpotential is required for the reaction.

Hydrogenase model compounds also provide support for understanding the enzymatic mechanism. A model compound of the [FeFe]-hydrogenase active site (Figure 1-8, panel b), has different protonation states, bearing a thiolate proton and a Fe hydride characterised by FTIR, crystallography and NMR. The protonated products of this complex depict the key intermediates in the catalytic mechanism for hydrogen production.²²

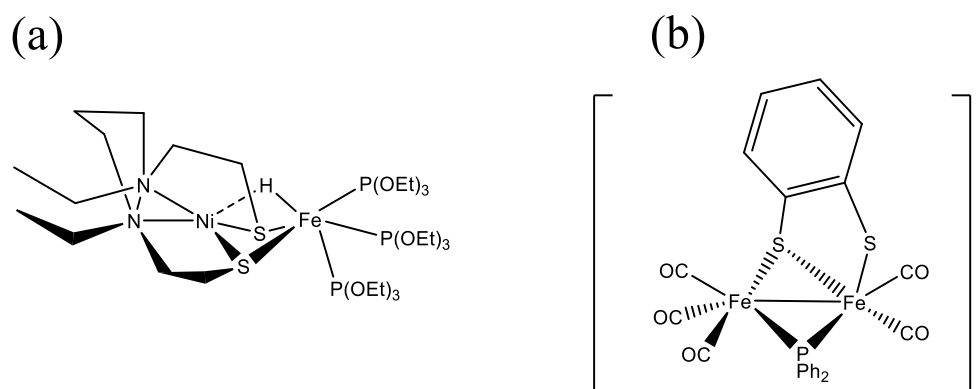


Figure 1-8. Biomimetic compounds designed for: (a) mimicking the catalytic activity of [NiFe]-hydrogenases;³ and (b) understanding the enzymatic mechanism of [FeFe]-hydrogenases.²²

Hydrogenase model compounds incorporated into protein frameworks

Recently, three synthetic complexes (with different bridging dithiolate ligands (Figure 1-9, panel a) are reported to be capable of being inserted into bacterial *Thermotoga maritima* apo-HydF (lacking the bimetallic active site of [FeFe]-hydrogenases). It was shown that the cluster could then be transferred to apo-HydA1 spontaneously to assemble the H-cluster of [FeFe]-hydrogenases (Figure 1-9, panel b and panel c). More interestingly, only the mimic with the amine containing dithiol ligand (adt) produces an active hydrogenase.²³ The identity of the bridgehead atom of the dithiolate ligand has been debated and remained uncertain for a long time, but this finding provides strong evidence that the adt is the ligand in the native enzyme.

The important role of the protein scaffold in H₂ activation was further pointed out by Hu and co-workers. The Hu group synthesised a model complex reproducing the structural features of [Fe]-hydrogenases with a five-coordinate iron center.²⁴ This mimic shares close spectroscopic properties and exhibits similar behaviour towards CO binding to the [Fe]-hydrogenases. However, this complex does not react with H₂. Hu and co-workers further conducted DFT calculations to elucidate what factors make such a difference compared with the native enzyme. The results suggested that binding of H₂ to the synthetic complex is unfavorable by about 10 kcal mol⁻¹, but once the H₂ is bound, the heterolytic cleavage of H₂ is facile.²⁵ Furthermore, for their projects in collaboration with Dr. S. Shima, some of Hu's model compounds can be incorporated into the apoenzyme of [Fe]-hydrogenases, and the generated

artificial enzymes have catalytic activities.²⁶ The ability of H₂ activation in these artificial enzymes might be contributed by the protein scaffold, which is able to get H₂ in place to facilitate the binding of H₂ with the active site. The significant difference in H₂ activation between the model complexes and the artificial enzymes provides fruitful hints for the design of future biomimetic catalysts.

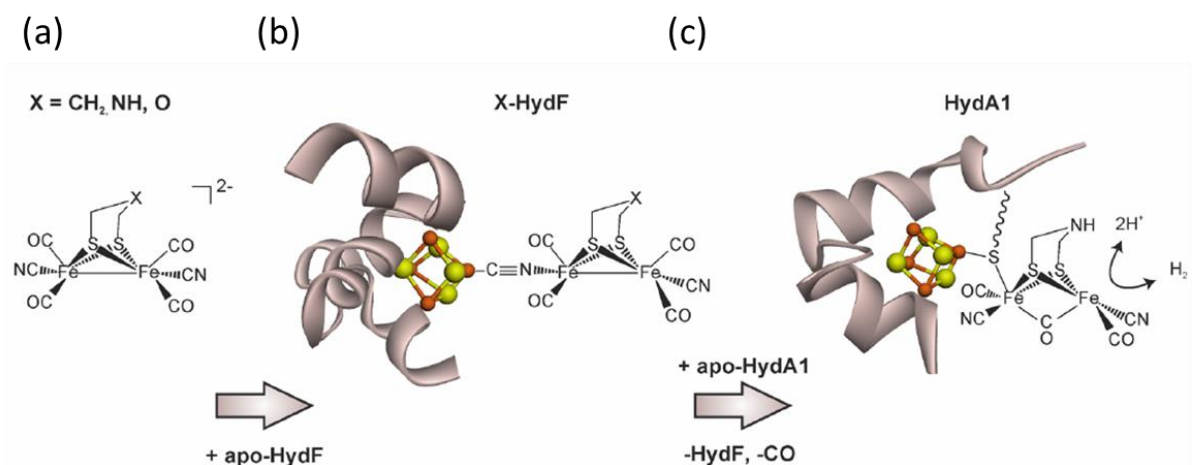


Figure 1-9. An artificial maturation of the H-cluster in a [FeFe]-hydrogenase; panel (a), synthetic mimics with different bridgehead atoms in the dithiolate ligand used for insertion; panel (b), a proposed structure of the hybrid protein (synthetic mimics bound to the apo-HydF); and panel (c), the H-cluster (active site) is formed by dissociation of one CO ligand, which can be detected in the haemoglobin based assay. Figures are adapted and reprinted with permission from ref 8, Copyright © 2014, American Chemical Society

1.2 Classification of [NiFe]-hydrogenases on the basis of biological functions

[NiFe]-hydrogenases were classified into four main phylogenetic groups (Table 1-1) on the basis of sequence analyses by Vignais and Billoud.¹¹ But recently, a novel group of [NiFe]-hydrogenases termed group 5 has been defined due to the distinct genes (compared with those in group 1-4) responsible for encoding a putative [NiFe]-hydrogenase.^{27,28}

Table 1-1. The classification of [NiFe]-hydrogenase groups.^{11,27,28}

Group	Function	Example
1	Membrane-bound H ₂ uptake hydrogenases	<i>E. coli</i> hydrogenase 1 and 2, <i>R. eutropha</i> H16 MBH
2a	Cyanobacterial H ₂ uptake hydrogenases	<i>L. aestuarii</i> CCY 9616
2b	Hydrogen sensors	<i>R. eutropha</i> H16 RH
3a	F420-reducing hydrogenases	<i>M. marisnigri</i> JR1
3b	NAD-reducing hydrogenases	<i>P. horikoshii</i> OT3
3c	Methyl viologen-reducing hydrogenases	<i>S. fumaroxidans</i> MPOB
3d	NADP/NAD-reducing hydrogenases (Bidirectional)	<i>R. eutropha</i> H16 SH
4	H ₂ evolving hydrogenases (Energy conserving, membrane associated)	<i>E. coli</i> hydrogenase 3
5	High affinity of the enzyme towards H ₂ to catalyse H ₂ oxidation at atmospheric levels	actinobacterial hydrogenase (AH) of <i>R. eutropha</i>

Group 1 consists of membrane-bound respiratory hydrogenases, which couple H₂ oxidation to redox components having higher redox potentials than the

H⁺/H₂ couple, like NO³⁻, SO₄²⁻, and fumarate (anaerobic respiration) or O₂ (aerobic respiration). Hence, these hydrogenases are tuned to be H₂ consumers. The [NiFe]-hydrogenase 1 and 2 from *E. coli*, studied in this thesis, both belong to Group 1.

Group 2 is comprised of H₂ uptake (group 2a) and H₂ sensing (group 2b) hydrogenases. Sequence analyses show that group 2a and 2b have a number of identical deletions in the primary amino acid sequences compared to those of group 1. Group 2b comprises the regulatory hydrogenases, acting as H₂ sensors in a signal transduction cascade to control hydrogenase gene transcription in response to H₂.

Group 3 consists of the dimeric hydrogenase modules, associated with other subunits for binding with soluble cofactors, e.g. F420 (8-hydroxy-5-deazaflavin), NAD or NADP. Group 4 is comprised of energy-conserving, membrane-associated enzymes. They can remove excess electrons produced by the oxidation of small organic compounds, e.g. CO or formate, by proton reduction. *E. coli* hydrogenase 3 is the prototype of this group. [NiFe]-hydrogenases which can perform H₂ oxidation at atmospheric levels, contributed by their high affinity towards H₂, are classified into group 5. They are found in soil-living actinobacteria and have clearly distinct genes for encoding the hydrogenase proteins compared with other groups. The actinobacterial hydrogenase (AH) of *R. eutropha* is a typical group 5 hydrogenase.

1.3 The architecture of [NiFe]-hydrogenases

A highly optimised functional protein matrix has been evolved for [NiFe]-hydrogenases. The catalytic centre is located in a hydrophobic pocket, which is deeply buried inside the protein. Substrates are transported via specific routes to facilitate efficient catalysis, as addressed below individually.

1.3.1 Active site

As mentioned in section 1.1.2, the active site of a [NiFe]-hydrogenase is located in the large subunit and is composed of Ni and Fe. Four cysteine sulphurs bind the bimetallic centre to the protein; two of these cysteine residues bridge the bimetallic site, and the remaining two coordinate to the Ni in a terminal fashion. The Fe is further coordinated by strong-field diatomic ligands, CO and CN⁻ (see Figure 1-3, panel b) and stays as low spin Fe^{II} during the catalytic cycle. The intrinsic CO and CN⁻ ligands in the active site are supposed to finely tune the metal centres electronically for facilitation of the ability to accept hydride formed during catalysis.

1.3.2 Iron-sulphur clusters

Since the catalytic centre of a [NiFe]-hydrogenase is deeply buried inside the protein, it relies on an electron relay chain of FeS clusters for electron mediation with its redox partner. In the case of standard [NiFe]-hydrogenases (or so-called O₂-sensitive [NiFe]-hydrogenases), the small subunit accommodates the proximal and distal [4Fe4S] clusters (with respect to the

bimetallic centre), and a medial [3Fe4S] cluster, positioned 12-14 Å apart (see Figure 1-10, panel a). It has been suggested that this distance range allows effective electron transfer without limiting the rate of catalysis of an enzyme.²⁹ In O₂-tolerant [NiFe]-hydrogenases, a modified proximal cluster in a [4Fe3S] configuration is found, and will be addressed in more detail in section 1.6.

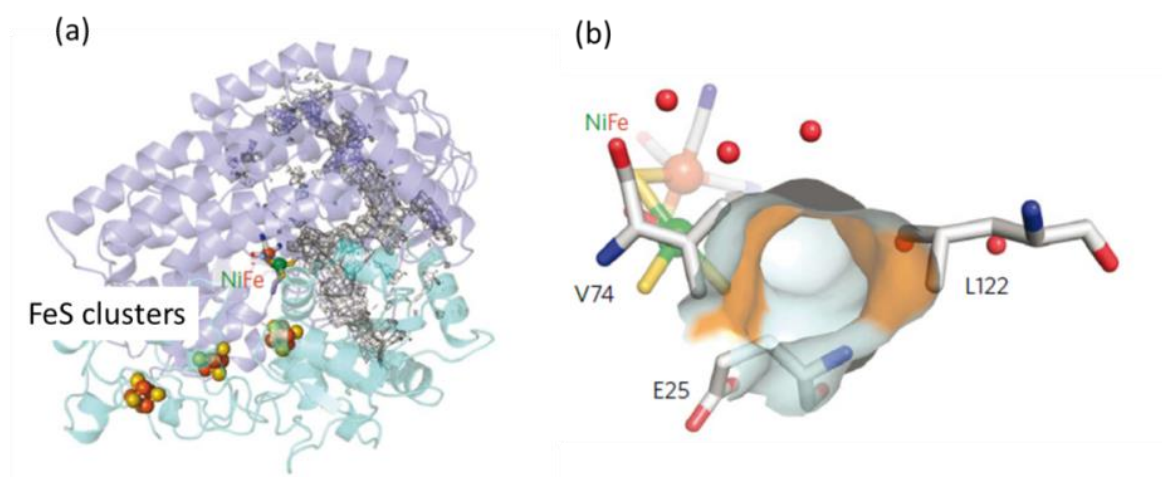


Figure 1-10. (a) Representation of the crystallographic structure of [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* (PDB code: 1YQW). The protein is shown in ribbon form, and the metal centres are shown as spheres. The grid delineating internal regions depict the hydrophobic gas tunnels. (b) Representation of the hydrophobic gas channel exit close to the active site. Figures are adapted and reprinted with permission from ref ³⁰. Copyright © 2009, Rights Managed by Nature Publishing Group.

1.3.3 Gas channels

The protein matrix is equipped with specific tunnels to guide gaseous substrate, i.e. H_2 , into the buried active site, but other common non-polar inhibitors of hydrogenases, such as O_2 and CO, can also be transported via these routes. Experimentally, existence of hydrophobic cavities for gas transportation in [NiFe]-hydrogenases was revealed by xenon-infused crystal structures and molecular dynamics simulations. These studies show that there are several hydrophobic gas channels in a hydrogenase molecule, merging near the Ni in the active site.³¹ In the case of [NiFe]-hydrogenase of *Desulfovibrio fructosovorans* (see Figure 1-10, panel a), there is a network of hydrophobic residues forming a Y-shaped channel around 30 Å long with two residues of Val74 and Leu122 constituting a narrow bottleneck at the entrance to the active site (see Figure 1-10, panel b). These two residues near the active site are conserved among O_2 -sensitive [NiFe]-hydrogenases. Mutagenesis studies show that the replacement of Val74 and Leu122 to more bulky residues of methionine in *Df* [NiFe]-hydrogenase improves its tolerance to O_2 , contributed by the slow diffusion rate of gases getting into the active site. However, more detailed studies by site-directed mutagenesis and a computational approach reveal that the size of bottleneck of the gas channel is not a crucial factor for O_2 tolerance.^{32,30,33}

1.3.4 Proton transport

As highly efficient catalysts for dihydrogen activation, hydrogenases are equipped with appropriate proton transfer pathways to remove the proton produced by heterolytic cleavage of H_2 quickly. Studies of hydrogenase model compounds suggest that this is one of the key points for mimicking a high turnover frequency.³⁴ In the case of [NiFe]-hydrogenases, the mechanism of proton transfer has not been completely elucidated yet, and several pathways have been proposed by computational and mutagenesis approaches. One proposed route is that one of the terminal cysteines coordinating to the Ni is the first position to accept protons, and the protons are further transferred either to the C-terminal metal site via ordered water molecule networks or to the molecular surface through the Glu chains which are located near the proximal FeS cluster.^{35,36} Another proton transport pathway going through the residue of Arg located opposite to the bridging hydride ligand, and then reaching the protein surface via His-rich motif has also been proposed.³⁷

1.4 Techniques for studies of hydrogenase chemistry⁷

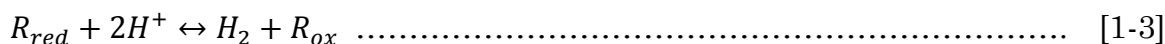
All understanding about hydrogenases so far was revealed by various methods, as addressed below individually.

1.4.1 Assays of activity

The study of hydrogenase mechanisms was started with different assay methods, which measure directly or indirectly how rapidly hydrogenases interact with their substrate, H₂ or protons.

Spectrophotometric assay

A standard method to quantify the activity of hydrogenases is to follow the colour change of electron acceptor/donor for H₂ consumption/production by spectrophotometry, as shown in [1-2] and [1-3], where A denotes a two-electron acceptor, and R represents a two-electron donor. For instance, in the case of oxidation of molecular hydrogen, methyl viologen is often used, which turns blue on reduction.



Conversion and exchange reaction

H₂ exists in different isotopic and nuclear spin forms. Conversion of para H₂ (*p*H₂, which has spin state (↑↓)) to ortho H₂ (*o*H₂, with spin state (↑↑)) by hydrogenases is observed in H₂O, and can be detected by the changes in

thermal conductivity.³⁸ In pure D₂O, the exchange reaction with H₂ produces HD and DD, with HD as a dominant product (probed by mass spectroscopy).^{39,40}

These investigations suggest that hydrogen is activated heterolytically to form a hydride and a proton, as written in equation [1-4], where E represents the enzyme.



Conversion in H₂O can be represented as:



The exchange reaction can be shown as:



In contrast to hydrogenases, a catalyst used industrially in fuel cells for oxidation of molecular hydrogen, platinum, catalyses homolytic cleavage of H₂, as the exchange reaction in D₂O produces DD predominantly.⁴¹

1.4.2 X-ray Crystallography

Crystal structures provide direct information on three-dimensional atomic architectures of hydrogenases. Structural information is the basis for further studies of catalytic mechanisms. In this technique, a three-dimensional view of the electron density is obtained by Fourier analysis of diffraction data first, followed by fitting atoms within the electron density maps to build molecular architecture models.

The major challenge in this method is to obtain large and well-ordered crystals, which are directly related to qualities of electron density maps derived from diffraction patterns for fine resolution and accurate structural determination. Moreover, other problems in analysing structural aspects are the difficulty in obtaining crystals in definable and homogeneous oxidation states. State transitions are hard to follow and hydrogen species are too light to be easily identified directly by X-ray diffraction.

1.4.3 X-ray absorption spectroscopy (XAS)

Limited structural information is able to be extracted in a more flexible way from X-ray absorption spectroscopy (XAS). In sharp contrast to X-ray crystallography, which requires a single crystal for obtaining proper diffraction patterns to resolve a complete three-dimensional structure of a molecule, XAS can probe a variety of sample types, like amorphous solid samples, frozen solutions, solutions, and gases. It is an element-specific and local bonding-sensitive spectroscopic technique.

This method requires an intense source (such as synchrotron light) and a tunable X-ray beam for dealing with dilute samples (millimolar). The spectrum arises from the absorption of ionizing radiation near an absorption edge. The edge is the abrupt absorption that occurs when a core electron is removed from the element of interest. The name of the absorption edges are given according to the principle quantum number, n , of the excited electrons, i.e. $n=1$ for K edge, $n=2$ for L edge, etc. There are three main regions found in a spectrum generated by XAS data: (i) a sharp rise, called the X-ray absorption edge, occurs at a well-defined X-ray photon energy, which is unique to the absorbing element, and is sensitive to oxidation states (higher oxidation states result in absorption at higher energies; (ii) a pre-edge region, resulting from bound state transitions. In the case of the K edges of first row transition metals, the pre-edge structures arise from the $1s$ to $3d$ transition when the $3d$ orbitals are not fully occupied. Since this transition breaks the selection rule ($\Delta l = \pm 1$, $\Delta s = 0$), the appearance of this transition is contributed by $3d$ and $4p$ orbital mixing and direct

quadrupolar coupling. Thus, the pre-edge region can provide information about to what extent the geometry of the absorption site distorts away from a centrosymmetric geometry. Spectral features in region (i) and (ii) are the so-called analyses of X-ray absorption near-edge structure (XANES). (iii) In the region at higher energy than the edge, photoelectrons ejected from the absorbing atom (denoted as a) interact with electron density of surrounding atoms (denoted as s), giving rise to damped sine waves of measurable frequency (related to distance between a and s), amplitude (related to coordination number about a and s atom types), and phase (related to s atom type). The analysis of spectral feature in this region is known as extended X-ray absorption fine structure (EXAFS).

XAS has been used mostly for [NiFe] hydrogenases, as the information specifically pertains to the Ni site, whereas Fe XAS spectrum would represent an average of the twelve Fe centres (including the Fe atoms in the electron relay chain) present in the protein. Valuable information obtained from this technique for understanding [NiFe]-hydrogenases is that a planar Ni site was not observed in any of the states of the enzyme; it is more likely that the coordination number of the Ni would be either five or six.

1.4.4 Mössbauer spectroscopy

Mössbauer spectroscopy probes transitions within an atomic nucleus, and requires the emission of γ rays from the source element in an excited nuclear state, and absorption of these by the same element in the sample under study. The Mössbauer effect requires that the absorption and emission of the γ radiation take place in a recoil-free manner, which is accomplished by placing the nuclei in a solid or frozen solution matrix. In hydrogenase research, ^{57}Fe element is used in this technique. Changes in the energy required for absorption are contributed by three main types of interaction of the nuclei of interest, i.e. an iron nucleus, with its nearby chemical environment: (i) isomer shift, δ , which is a measure of the s -electron density on the nucleus, yielding information on oxidation state, spin state, coordination sphere of the metal ion, and the degree of covalency in the metal-ligand bond; (ii) electric quadrupole interaction, a nucleus with a spin quantum number $I > \frac{1}{2}$ has a quadrupole moment tensor Q , due to the non-spherical charge distribution. This asymmetric charge distribution around the nucleus causes an asymmetric electric field at the nucleus, characterised by a tensor quantity called the Electric Field Gradient (EFG), ∇E . The electric quadrupole interaction between these two quantities gives rise to a splitting in the nuclear energy levels. This interaction reveals information on oxidation state, spin state and symmetry of the electric field of the nucleus of interest; (iii) magnetic hyperfine interaction, a nucleus with spin quantum number I has $(2I+1)$ magnetic energy levels, causing splitting in Mössbauer spectra.

1.4.5 Electron paramagnetic resonance (EPR) spectroscopy

EPR spectroscopy is exploited to study systems with one or more unpaired electrons. The origin of an EPR signal is the energy splitting of electron spin states, contributed by the spin moment ($m_s = \pm 1/2$ for a free electron) of unpaired electrons interacting with an applied magnetic field, which is termed the Zeeman effect. There are two effects contributing to changes of magnetic field experienced by the unpaired electrons: (i) spin-orbit coupling, which arises from a moving electron in orbit about a nucleus, which generates an orbital momentum (L) adding to the magnetic field experienced by the electron spin (S); and (ii) zero-field splitting, which occurs when there is more than one unpaired electron in the paramagnetic centre, causing the energy separation of the various m_s states in the absence of an external magnetic field. Moreover, in respect of the unpaired electron, when there is a nucleus with nuclear spin nearby, such a magnetic interaction, namely the hyperfine interaction, results in additional allowed energy states and, in turn, multi-lined spectra. The spacing between the EPR spectral lines indicates the degree of interaction between the unpaired electrons and the perturbing nuclei. Thus, a variety of information can be obtained by careful analyses of the spectroscopic parameters in hydrogenase research, such as the (formal) oxidation state and spin state of the metal, the d-orbital occupation, the symmetry of the crystal field, the spin distribution, or the exchange coupling with other metal centres. The combination of EPR and Mössbauer spectroscopies (carried out on parallel samples prepared carefully) makes a detailed characterisation of all the redox

states of the clusters present in the enzyme possible. And then, redox potentials of the FeS clusters of the electron relay chain can also be determined.

1.4.6 Protein film electrochemistry (PFE)

Protein film electrochemistry has been developed for studies of the redox activity of electron-transfer proteins. With the benefit of the direct immobilisation of proteins onto the electrode, sensitive measurements are made of currents induced in response to applied potentials, changes of substrate concentration and introduction of inhibitors. Thus PFE serves as a good tool in hydrogenase research for getting information, such as the oxidative active/inactive interconversion and the $H^+ : e^-$ stoichiometry of the reaction, complementing information provided by other methods (section 2.1.6 for more details).

1.4.7 Fourier transform infrared spectroscopy (FTIR)

The active sites of hydrogenases contain built-in biologically unusual ligands, CO and CN^- , which have intense infrared absorption features. The ν_{CO} and ν_{CN} stretching bands are in the $2200\text{-}1800\text{ cm}^{-1}$ region, which is clear of solvent and other biological ligand absorption features. Vibrational frequencies of these diatomic ligands are very sensitive to the changes in coordination and electronic environment of the active site, allowing conclusions about changes of redox states and to identify inhibited species. The sample handling for IR measurement is easily adapted for physiologically relevant conditions, i.e. in pH neutral solution, and at room temperature and under ambient pressure.

This complements information available from other techniques with inherent limitations, for example, the difficulties that exist in getting definable and homogeneous oxidation states in crystallography, and the fact that electron paramagnetic resonance (EPR) spectroscopy can only be applied to probe paramagnetic states of biological iron sulphur clusters in a frozen condition (*vide supra*). Moreover, FTIR is easy to use in conjunction with other methods, such as stopped-flow techniques and electrochemistry (see below).

1.4.8 Protein film infrared electrochemistry (PFIRE)

FTIR is one of the techniques that is easy to be coupled with electrochemistry to ascertain potential-dependent structural information. The spectroelectrochemical technique used throughout this Thesis is protein film infrared electrochemistry (PFIRE), which exploits a particle electrode with immobilised redox enzyme samples on the surface in conjunction with ATR-IR spectroscopy for *in situ* studies. In this technique, direct electrochemical control over the enzyme can be achieved, while a FTIR measurement is carried out (see section 2.5 for more details).

1.4.9 Density functional theory (DFT)

DFT was developed in 1964 by Hohenberg and Kohn,⁴² who demonstrated that ground state properties of a many-electron system were uniquely determined by an electron density (ρ), and proved that only the true electron density at ground state gave the lowest energy. Thus, computational demands for wavefunction determination are reduced to deal with three spatial coordinates only, which allows DFT for studies of bigger systems, such as transition metal compounds. In hydrogenase research, DFT is a powerful tool for analysis of spectroscopic results, such as data from FTIR and EPR, and also plays an important role in mechanistic studies. For example, the Ni-L state at a Ni^IFe^{II} redox level, which was thought to be irrelevant to the catalytic cycle, was proved experimentally to be an intermediate during the conversion of Ni-C to Ni-SI_a to restart the catalytic cycle very recently (see Chapter 4). However, a similar concept was proposed years ahead by DFT calculations from the work of Hall and co-workers^{43,44} and Lill and Siegbahn.⁴⁵

1.5 Complexity of redox states of [NiFe]-hydrogenases

Information on various active and inactive states has been suggested from a range of techniques, such as EPR, FTIR and PFE.^{7,8,46} Reaction of [NiFe]-hydrogenases (in the case of “standard” hydrogenases, *cf.* section 1.6) with O₂ gives rise to mixtures of at least two most oxidised inactive forms (Ni^{III}, *S*=1/2): Ni-A (unready) and Ni-B (ready). Ni-A had been assigned to have a bridging peroxide group between the metal ions in the active site, however, this is now thought not to be the case, and the identity of the bridging ligand still remains an open question.⁸ The latest argument on the structure of Ni-A is that it still bears a bridging hydroxide ligand, but one of the bridging thiolate ligands is oxidised to be either sulfenate or sulfenic acid.⁴⁷

Generally, a longer time is required for Ni-A to be activated relative to Ni-B; it usually takes several hours for activation in the presence of H₂.¹ Ni-B is believed to contain a bridging hydroxo ligand, and can be activated within seconds upon reduction.¹ After the oxygen-based bridging ligand is removed, four intermediates have been regarded as being directly involved in the catalytic cycle: Ni-SI_a, Ni-C, Ni-L (is included to the catalytic cycle very recently, see section 4.1) and Ni-R. Ni-SI_a is a Ni^{II}Fe^{II} species which has an open bridge with a four-coordinate Ni (see Figure 1-11), and thus it is considered as an initial state reacting with H₂ (see section 4.1 for more details about the catalytic cycle). One electron oxidation of Ni-SI_a can re-generate the Ni-B state, albeit this process is slow under anaerobic conditions.⁴⁸ One-electron reduction and

protonation of Ni-SI_a leads to Ni-C, with a hydride bridging two metals.⁴⁹ The Ni of Ni-C has a higher formal oxidation state (Ni^{III}) than that of the Ni-SI_a state (Ni^{II}), due to the electron density shifting away from the Ni to the bridging hydride. Hence, the Ni-C state is EPR-active. In most cases, photo-illumination of Ni-C at cryogenic temperatures generates states at a Ni^IFe^{II} redox level (also EPR-active), known as Ni-L (the exception occurs in some O₂-tolerant hydrogenases, see section 1.6 for more details). Usually, more than one Ni-L species are characterised spectroscopically, and are often referred to as Ni-L_I, Ni-L_{II}, etc.⁴⁶ The mechanism of Ni-L formation has been unveiled by detailed EPR studies,⁵⁰ which indicated that the photolysis of Ni-C makes the bridging hydride release as a proton (with the Ni formally being reduced to Ni^I), and the proton migrates to nearby amino acids. The existence of various Ni-L states implies that more than one site can accept this proton. Further DFT computational work from Neese and co-workers showed that the presence of a metal-metal bond (which is formed by an electron pair donated from Ni^I to Fe^{II}) in the Ni-L state,⁵⁰ and this bond serves as a base; protonation of this metal-metal bond gives rise to Ni-C. Therefore, although the formal oxidation states of the Ni are different between Ni-C and Ni-L, Ni-L can be regarded as tautomeric forms of the Ni-C state at the same oxidation level within the extended active site, i.e. the bimetallic centre and the nearby residues which serve as proton acceptors. Ni-L has generally been assumed to be irrelevant to the catalytic cycle, due to the requirement of specialised conditions for formation of this state. However, very recently, Ni-L has been proved as a

catalytic intermediate experimentally;^{51–53} it is an intermediate during the conversion of Ni-C to Ni-SI_a to restart the catalytic cycle via sequential proton- and electron-transfer steps (see Chapter 4 for more details).

One more electron reduction of Ni-C gives rise to the most reduced set of states, Ni-R at a Ni^{II}Fe^{II} redox level with a bridging hydride retained in the bimetallic centre.⁵⁴ More than one Ni-R species are often characterised spectroscopically, and are referred to as Ni-R_I, Ni-R_{II}, etc. Various Ni-R species probably also represent different protonation states within the extended active-site region, suggested from studies on a range of hydrogenases.⁴⁶ Figure 1-11 summarises the interconversions between active and inactive states of [NiFe]-hydrogenases, in which the Fe has only been detected in the oxidation state of II, whereas the formal oxidation states of I, II, and III have been identified on the Ni.

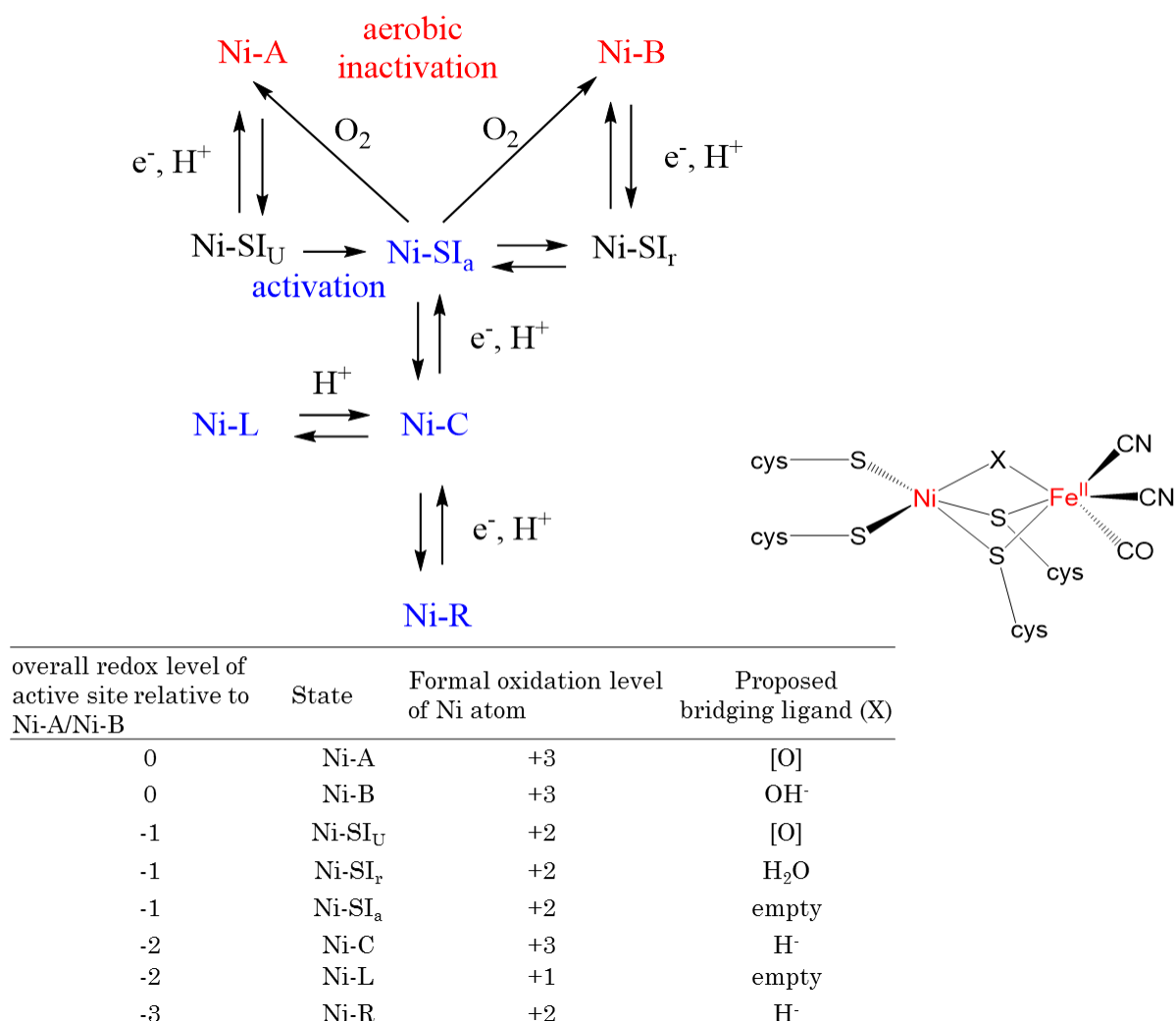


Figure 1-11. Spectroscopically characterised active and inactive states of [NiFe]-hydrogenases, represented by the structure of the active site.

1.6 O₂-tolerant [NiFe]-hydrogenases^{8,33,55,56}

Most [NiFe]-hydrogenases lose their activity upon contact with O₂. However, there is a special class of membrane-bound [NiFe]-hydrogenases which are active under aerobic conditions, termed O₂-tolerant MBH. This is in contrast to the general type of [NiFe]-hydrogenases, which are strongly inactivated by O₂ and called “standard hydrogenases” or “O₂-sensitive hydrogenases”. O₂-tolerant MBHs are found in a range of organisms, such as: Knallgas bacterium, *Ralstonia eutropha*; the aerobic thermophile *Aquifex aeolicus*; and the enteric bacterium *Escherichia coli*.

The most distinct feature of O₂-tolerant MBHs from standard hydrogenases is that the majority state formed upon encountering O₂ is Ni-B. Ni-B is the inactive state which can be reactivated quickly by one electron reduction to bring the enzyme back to the catalytic cycle (see section 1.5), and thus a rapid formation of this protective state is the crucial factor for O₂-tolerant MBHs keeping their activities in the presence of O₂. This process requires rapid and successive electron transfers to reduce the O₂ into water and hydroxide (which is a four-electron, three-proton process, *vide infra*), and thus O₂-tolerant MBHs have been re-termed oxygen reductases in a revised classification⁵⁵ due to such a distinctive property.

FTIR and crystallographic studies indicate that the active site structures of O₂-tolerant MBHs are similar to those in standard hydrogenases (see Figure 1-3).^{51,57–59} Their unusual abilities to reduce O₂ rapidly are contributed by the

specialised FeS clusters of the electron relay chain in two respects: (i) the redox potentials of the three FeS clusters are all considerably higher (more positive) than those for their counterparts in the standard hydrogenases, which are finely tuned for reacting with O₂ (see Table 1-2 for comparison); and (ii) the proximal cluster has a unique structure in a [4Fe3S] configuration, which is able to transfer two electrons to the active site sequentially at similar potentials. Figure 1-12 depicts the structure of the [4Fe3S] proximal cluster from an O₂-tolerant MBH, demonstrated by the crystal structure of [NiFe]-hydrogenase 1 (Hyd-1) from *E. coli*.⁵⁷ This cluster is ligated by six cysteine residues; four of them are highly conserved in all [NiFe]-hydrogenases, and thus the other two additional cysteine residues, are usually called the supernumerary cysteines (Cys19 and Cys120 for *E. coli* Hyd-1). The thiolate of Cys19 substitutes for one sulphide, resulting in the architecture of [4Fe3S]. In the reduced state ([4Fe3S]³⁺), this cluster stays in a [4Fe3S] cubane-shape (Figure 1-12, panel a). However, in the super-oxidised state ([4Fe3S]⁵⁺), the deprotonated amide nitrogen of Cys20 coordinates to the Fe4, inducing a conformational change by breaking the bond between the Fe4 and the sulphide (Figure 1-12, panel b). The super-oxidised state, which contains three ferric Fe ions and one ferrous Fe ion, can be stabilised by this structural change, as the deprotonated amide nitrogen of Cys20 compensates the cluster system for a negative charge via forming a new chemical bond.⁵⁷

Table 1-2. Midpoint potentials of the FeS clusters in O₂-tolerant MBHs and standard hydrogenases.^{a,b}

	O ₂ -tolerant MBH		
	<i>E. coli</i> (pH=6)	<i>R. eutropha</i> H16 (pH=7)	<i>R. metallidurans</i> CH34 (pH=7)
[4Fe3S] ^{5+/4+} <i>proximal</i>	+ 230	+ 160	+ 240
[4Fe3S] ^{4+/3+} <i>proximal</i>	+ 30	-	-
[3Fe4S] ^{+/0} <i>medial</i>	+ 190	+ 25 <i>app</i>	+ 100 <i>app</i>
[4Fe4S] ^{2+/+} <i>distal</i>	-	-60	50
reference	60	61	61
	standard hydrogenase		
	<i>D. vulgaris</i> MF (pH=7.7)	<i>D. gigas</i> (pH=7)	<i>D. fructosovorans</i> (pH=8)
[4Fe4S] ^{2+/+} <i>proximal</i>	< -300 <i>app</i>	- 290 <i>app</i>	- 340 <i>app</i>
[3Fe4S] ^{+/0} <i>medial</i>	- 70 <i>app</i>	- 70 <i>app</i>	+ 65 <i>app</i>
[4Fe4S] ^{2+/+} <i>distal</i>	< -300 <i>app</i>	- 340 <i>app</i>	- 340 <i>app</i>
reference	62	63	64

^aThe midpoint potentials are given in mV vs SHE.

^bThe apparent midpoint potential ('app') refers to the potential at which the uncoupled cluster signal is at half its maximum intensity

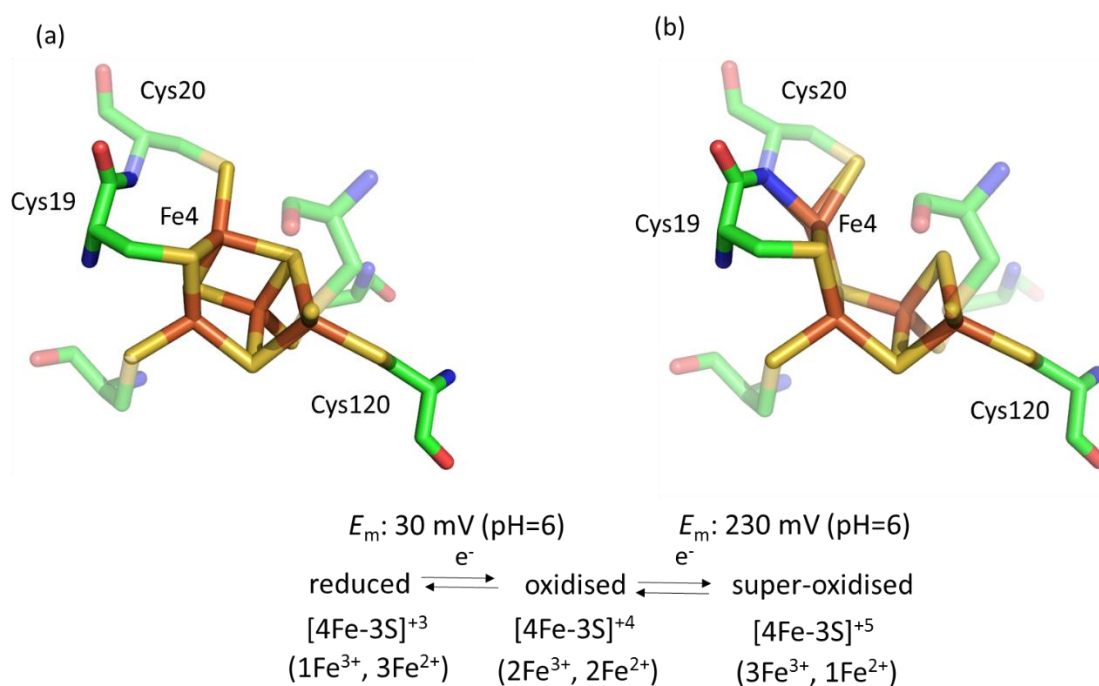


Figure 1-12. Structure of the Cys₆[4Fe₃S] proximal cluster existing among O₂-tolerant MBHs in the (a) reduced (PDB code: 3UQY) and (b) super-oxidised (PDB code: 3USC) redox states, demonstrated by the crystal structure of *E. coli* Hyd-1.⁵⁷ The four cysteine residues conserved among standard [NiFe]-hydrogenases are shown in 50% transparency. Midpoint potentials (E_m) of the two redox transitions of the proximal cluster for *E. coli* Hyd-1 at pH 6 are given.⁶⁰ Formal charges of Fe atoms of the cluster are shown in brackets.

States of Ni-SI_a, Ni-C, Ni-L and Ni-R are considered as species involved in the catalytic turnover (see section 1.5, and 4.1), in which Ni-SI_a is the most oxidised catalytic intermediate; it is one electron more reduced than the Ni-B state. If O₂ attacks the active site at the level of Ni-SI_a, the bimetallic core can provide an electron immediately in response to this O₂-binding event, generating a superoxide-like species. Thus, in order to form the protective state of Ni-B (with a hydroxide ligand bridging the bimetallic centre, see Figure 1-11), three more electrons from FeS clusters are required rapidly to reduce this highly reactive species into water and hydroxide. It should be noted that this is the maximum number of electrons needed to be supplied from the electron relay chain in this protective mechanism. If O₂ attacks at the level of Ni-C (or its isoelectronic state, Ni-L)/Ni-R, only two/one additional electrons are required for the rapid formation of Ni-B, because Ni-C (or Ni-L) and Ni-R are two and three electrons more reduced than the Ni-B state, respectively. The evolved FeS clusters of O₂-tolerant MBHs can satisfy this demand readily. The specialised [4Fe3S] proximal cluster is able to deliver two successive electrons, accompanied with a conformation change to form an unusual Fe–N(amide) to stabilise the cluster at a super-oxidised state ([4Fe3S]⁵⁺), *vide supra*. These two successive electrons reduce the superoxide moiety into a hydroperoxide species, then a water and a hydroxyl radical sequentially. The fourth electron is crucial to avoid the enzyme being trapped in the Ni-A state, which is provided by the medial cluster to reduce the hydroxyl radical, generating Ni-B. The significantly high redox potentials of the three FeS clusters in O₂-tolerant MBHs means that the

electron relay chain is far more likely to have electrons ready any time for rapid supply to the active site when it needs them under more oxidative conditions during catalytic turnover.

The specialised FeS clusters with unusually high redox potentials also lead to distinctive features in O₂-tolerant MBHs from standard hydrogenases: (i) a catalytic bias towards H₂ oxidation (at neutral pH); and (ii) the requirement for a characteristic overpotential of 50-100 mV at neutral pH (depending on the hydrogenase) to drive the H₂ oxidation. These properties can be rationalised well by a mathematical model, which separates energy input (thermodynamics and electrochemical rates) from the reversible chemical steps of catalysis, from the work of Hexter *et al.*⁶⁵ The results indicated that the overpotential requirement and catalytic bias of an enzyme immobilised on an electrode are governed by the redox potential of the entry point at which electrons enter or leave the catalyst, with respect to the equilibrium potential of the reaction being catalysed. In the case of [NiFe]-hydrogenases, the distal [4Fe4S]^{2+/+} cluster is the gate at which electrons enter or leave the enzyme during H⁺/H₂ cycling. Thus, the smaller redox potential difference between the distal cluster and the H⁺/H₂ couple is, the more reversible and efficient the electrocatalyst. From studies of the O₂-tolerant MBH *R. eutropha* H16 by Knuttel *et al.*,⁶¹ the midpoint potential of the distal [4Fe4S]^{2+/+} cluster is -60 mV at pH 7, which is far more positive relative to the H⁺/H₂ redox potential (-413 mV at pH 7; 1 bar H₂; 25°C), and is also more than 200 mV more positive than those for the counterparts in standard hydrogenases, i.e. -340 mV for *D. gigas*⁶³ at pH 7

(cf. Table 1-2). For another O₂-tolerant MBH, *E. coli* Hyd-1, the model for electrocatalysis predicted an entering potential of -190 mV at pH 6 (as the actual potential could not be determined by titrations in EPR),⁶⁵ which is also far above the H⁺/H₂ couple of -353 mV (pH 6; 1 bar H₂; 25°C). This means that the distal cluster holds the electron tightly, hindering the proton reduction reaction. Also, it requires an applied potential which must be positive enough to oxidise the distal cluster for initiating electron flows to drive the reaction of H₂ oxidation. However, since the potential of the [4Fe4S] clusters shows negligible pH-dependent effects, while the redox potential of H⁺/H₂ couple shifts by -60 mV per pH unit and by -30 mV per order of magnitude increase in H₂ pressure, work from Murphy *et al.*⁶⁶ demonstrated that *E. coli* Hyd-1 is able to be transformed into a proficient, reversible H₂ producer below pH 4.

There are other characteristic properties shared among O₂-tolerant MBHs, which might be contributed by the subtle variations in the electronic environments and basicity of proton acceptors in the extended active-site region: (i) they are much more resistance to CO, a strong inhibitor of standard [NiFe]-hydrogenases;^{67,68} (ii) the bridging hydride retained in the bimetallic centre is weakly bound. The Ni-L states can form without illumination at cryogenic temperatures detected by EPR in the case of *A. aeolicus* Hase I⁶⁹ and *E. coli* Hyd-1⁶⁰. Further studies on *E. coli* Hyd-1 showed that there is a pH-dependent equilibrium between the Ni-C and Ni-L states, indicating that the proton and electron transfer steps need not be coupled for the conversion

from Ni-C to Ni-SI_a, in which the Ni-L states can be even detected by FTIR in the dark at room temperature (see Chapter 4 for more details).^{51,52}

1.7 Hydrogenases in *Escherichia coli*

The enteric facultative anaerobe, *Escherichia coli*, has at least three well characterised [NiFe]-hydrogenases, termed Hyd-1, Hyd-2 and Hyd-3 (see Figure 1-13).⁷⁰ Their properties can be understood with respect to overall metabolism of the organism. The former two enzymes are periplasmic membrane-bound proteins, and are expressed under microaerobic and anaerobic conditions, respectively. Hyd-1 contains an integral membrane b-type cytochrome protein (HyaC), which anchors the hydrogenase into the membrane (Figure 1-13, panel a). Under conditions of slow growth and fluctuating O₂ concentrations, Hyd-1 scavenges H₂, and the H₂ oxidation is coupled to the quinone reduction via the integral membrane protein, generating a proton gradient.^{8,67,71} Thus, a reverse electron transport from the quinone/quinol pool would break down the transmembrane electrochemical gradient. On the basis of this physiological feature, it is expected that Hyd-1 is completely biased towards being a strong H₂ consumer in *in vitro* studies.⁶⁷ As for Hyd-2, although it is also categorised as a group 1 hydrogenase (membrane-bound respiratory hydrogenases, see section 1.2), it works in a different way. According to the sequence analyses, Hyd-2 is predicted to be a large tetrameric complex consisting of a large (HybC) and a small (HybO) subunits associated with two other subunits: HybA, which is comprised of four

FeS clusters for electron mediation, and an integral membrane protein, HybB, comprising 10 transmembrane helices. The whole architecture of Hyd-2 is closely related to the [NiFe]-hydrogenase from *D. vulgaris*.¹¹ Physiologically, Hyd-2 is not coupled to a transmembrane HyaC-type quinone reductase. It exchanges electrons with the quinone/quinol pool without altering the proton gradient. Thus, if the quinone/quinol pool gets over-reduced, Hyd-2 can accept extra electrons to produce H₂, balancing the redox potential of the cell. Based on this physiological feature, it is envisaged that Hyd-2 is a bidirectional hydrogenase *in vitro*.⁶⁷

Hyd-3 is an O₂-sensitive component of the cytoplasmic membrane-bound formatehydrogenlyase complex, which catalyses the conversion of formate (produced during fermentation) to CO₂ and H₂, keeping a stable acid-base balance in the cytoplasm, and allowing for dihydrogen recycling. The whole complex comprises seven subunits: FdhF (the molybdenum-dependent formate dehydrogenase-H), HycE (termed Hyd-3, a [NiFe] hydrogenase component), three electron transfer proteins (HycB, HycF, and HycG) between FdhF and HycE, and two integral membrane proteins (HycC and HycD) for anchoring the complex into the membrane.⁷²

The subjects which are studied and discussed throughout this Thesis are Hyd-1 and Hyd-2. Hyd-1 is an O₂-tolerant MBH, whereas Hyd-2 behaves like a standard [NiFe]-hydrogenase in many aspects, such as: (i) it is strongly inactivated by O₂; (ii) the enzyme is inhibited by CO strongly; and (iii) Hyd-2

is an efficient and reversible catalyst for H_2 cycling without significant overpotential.⁶⁷

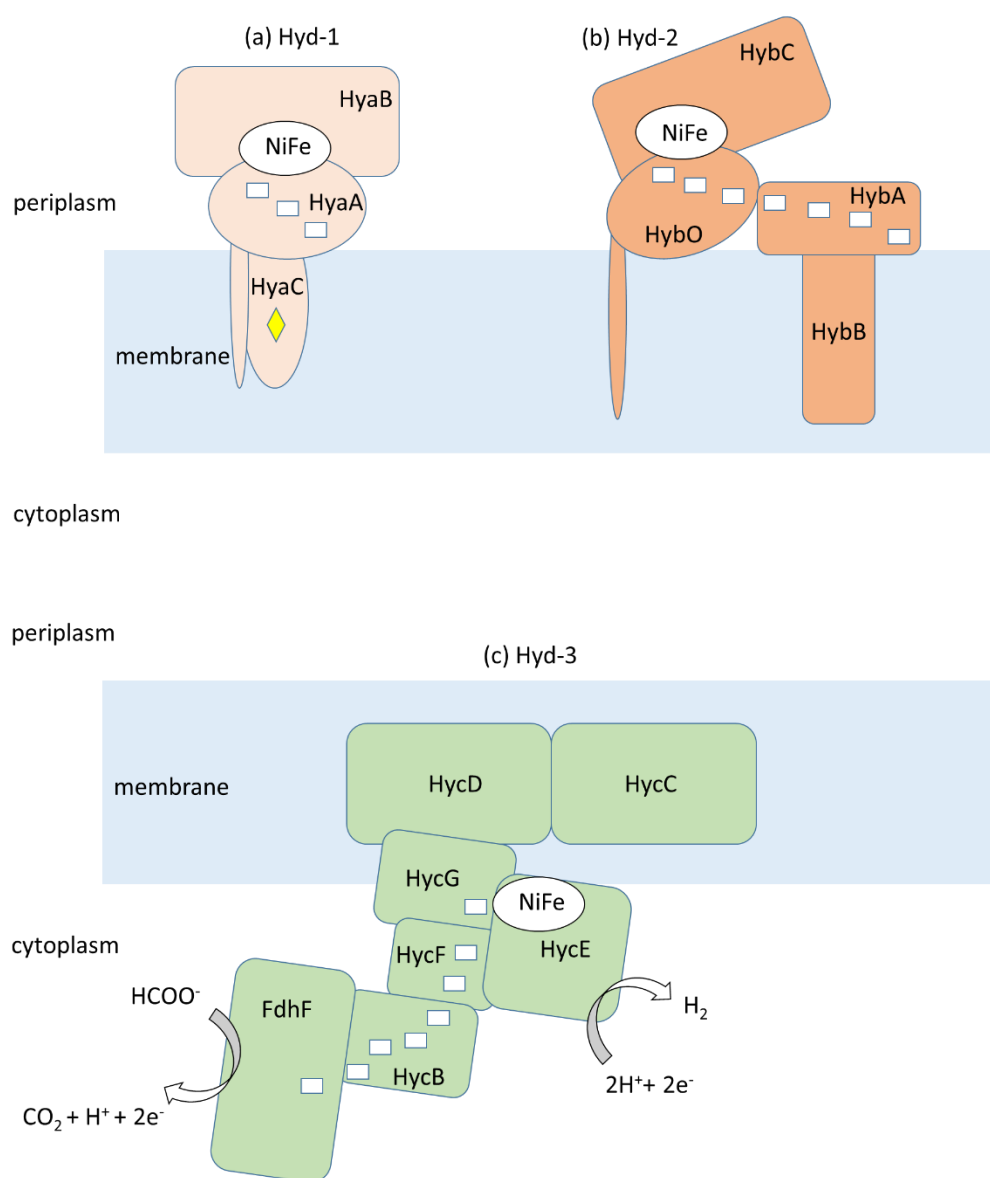


Figure 1-13. Cartoon representations of three native hydrogenases in *E. coli*. FeS clusters are represented by squares, and the haem b group is symbolised by a diamond.

1.8 Inhibition of [NiFe]-hydrogenases

1.8.1 CO

CO is a common inhibitor of hydrogen-cycling catalysts, especially precious metal based catalysts like Pt, to which harsh oxidative treatment is required for CO detoxification. In contrast, [NiFe]-hydrogenases are more resistant to CO; their activities can be recovered readily by just simply removing CO.^{1,67}

Detailed studies on CO inhibition of [NiFe]-hydrogenases by PFE showed that CO serves a competitive inhibitor to H₂, and imposes inhibition almost across the whole potential window on H₂ oxidation and production.^{8,67,73} The Ni-SI_a state was reported to be the target for CO binding, and a crystal structure of this inhibited species was also isolated and determined, showing that CO coordinates to Ni (see Chapter 6 for more details).⁷⁴

1.8.2 H₂S

Sulphide was also found to inhibit some [NiFe]-hydrogenases from sulfate reducing bacteria (such as *Desulfovibrio* species) and purple sulphur bacteria (such as *A. vinosum*).⁷⁵ Crystal structures with a bridging ligand interpreted as sulphur were reported by Higuchi et al.,⁷⁶ and H₂S gas could be detected upon activating the as-isolated hydrogenase with H₂ and electron donors.⁷⁷ Further studies by PFE indicated that this inhibition happens in a narrow potential window (above +100 mV at pH 6), and is easy to be missed out without precise potential control.⁷⁵ Although this potential window is hard to be

experienced *in vivo*, it is likely that this sulphur-trapped state is produced during aerobic purification.

1.8.3 KCN

Cyanide is known as a strong ligand coordinating to many transition metals and inhibiting several metalloproteins.^{78,79} However, only a few studies are reported on cyanide inhibition of [NiFe]-hydrogenases. In early studies on the inhibitory effect of cyanide towards [NiFe]-hydrogenase from *Azotobacter vinelandii*, cyanide was either applied to whole cell samples or to cell-free extracts,^{80,81} which showed that cyanide acts as an inhibitor to H₂ oxidation when O₂ is present, but there is no effect when cyanide is incubated with reduced, active hydrogenase under H₂. Further studies via spectrophotometric assay methods on purified hydrogenase of *A. vinelandii* showed that cyanide causes irreversible inhibition when the enzyme is at its oxidised state.⁸²

More detailed studies were conducted by Suzannah Hexter by PFE, in which the enzyme was directly adsorbed on an electrode.⁸³ The study of *E. coli* Hyd-2 showed that cyanide leads to a significant inhibition of H₂ oxidation when it is injected at high potentials, but this effect only occurs over a narrow potential window (above ca. -250 mV; pH 7). The inactivation was readily reversible when the applied potential was swept to lower values. The inhibitory effect of cyanide is not specific to *E. coli* Hyd-2, as *E. coli* Hyd-1 shares a similar behaviour revealed by PFE.⁸³

In work for this Thesis, an *in situ* technique called protein film infrared electrochemistry, PFIRE (see section 2.5), which couples direct electrochemistry with FTIR spectroscopy, is exploited to get more potential-dependent structural information on cyanide inhibition of *E. coli* Hyd-1 and Hyd-2 (see Chapter 5 for more details).

1.8.4 Isocyanide

Isocyanides are formally isoelectronic with CO and CN⁻, but are slightly better σ -donors and weaker π -acceptors than CO. Isocyanides have been reported as inhibitors of several metalloproteins, such as nitrogenase,⁸⁴ and carbon monoxide dehydrogenase (CODH),⁸⁵ and have been studied as CO analogues for haem proteins.⁸⁶ However, the effect of isocyanides on [NiFe]-hydrogenases has not been reported so far. This Thesis includes the first report of isocyanide inhibition for both *E. coli* Hyd-1 and Hyd-2 studied by PFE and PFIRE (see Chapter 7 for more details).

1.9 Summary and structure of this Thesis

Among H_2 oxidation catalysts with earth-abundant metal centres, hydrogenases are the most active. However, fundamentally, there are still a number of open questions in hydrogenase chemistry and catalytic mechanisms, demanding for a development of novel techniques to resolve ambiguities.

In this Thesis, a new approach, protein film infrared electrochemistry (PFIRE), is demonstrated for *in situ* studies on hydrogenases (see section 2.5 for detailed description), by which direct electrochemical control over the enzyme can be achieved, while a FTIR measurement is carried out. An essential prerequisite for applying this technique is that there must be enough enzyme molecules immobilised on the particle electrode to provide a good signal to noise ratio in spectra. Thus, in an attempt to apply this technique to *E. coli* Hyd-2, a concept of using surfactant-modified carbon nanoparticles to facilitate enzyme immobilisation is introduced and described in Chapter 3. A high loading of Hyd-2 on the surfactant-modified particles is contributed by the hydrophobic interaction, because Hyd-2 is a membrane-associated protein, which has a large footprint with hydrophobic feature interacting with the surfactant-modified particles. Also, a physiologically-mimicking environment can be reconstructed on surfactant-modified particles, allowing enzyme to adsorb on particles in a right orientation for effective electron transfer without causing significant overpotential in catalysis. A comparison of immobilisation

strategies between coupling reagent for covalent bonding and surfactant-modified particles will also be discussed in more detail in Chapter 3.

Following the successful strategy of Hyd-2 immobilisation for effective electron transfer, redox states of Hyd-2 are characterised by PFIRE and are presented in Chapter 4, which also addresses issues of what states are involved in the catalytic turnover, and compares the differences between Hyd-1 (O₂-tolerant MBH) and Hyd-2 (standard hydrogenase). Chapter 5 describes studies of the unusual reaction of [NiFe]-hydrogenases with cyanide; Chapter 6 presents results of CO inhibition of *E. coli* Hyd-1 and Hyd-2; and Chapter 7 represents the first report that isocyanides inhibit [NiFe]-hydrogenases reversibly, revealed by PFE and PFIRE.

Finally, Chapter 8 summarises all projects, and discusses the different responses to the substrate, H₂, and a series of π -acid ligands: cyanide; CO; O₂; and isocyanide, between Hyd-1 and Hyd-2, and then offers an outlook for further studies by means of the powerful approach, PFIRE, on hydrogenases.

Chapter 2: Theory and Methods

The main techniques exploited throughout this Thesis are electrochemistry and FTIR spectroscopy. Protein film electrochemistry (PFE) is used for electrochemical studies, in which hydrogenase samples are immobilised on the working electrode, and thus the catalytic currents correspond directly to the activities of enzyme, serving as a sensitive tool to unveil hydrogenase chemistry. The spectroelectrochemical technique of protein film infrared electrochemistry (PFIRE), which couples FTIR and direct electrochemistry together, is the method to ascertain potential-dependent structural information on hydrogenases in this Thesis. This chapter will describe the theory of each approach and the detailed experimental methods used throughout studies.

2.1 Basic theory of electrochemistry

2.1.1 Redox reaction

Reactions which are accompanied with transport of charges between species are called redox reactions. In an electrochemical cell, this process happens at the interface between an electrode and an electrolyte. A control of the potential of the working electrode (*with respect to* the reference electrode, which has a fixed potential) means to adjust the energy of the electrons within the working electrode, i.e. the energy of electrons is raised by driving the electrode to more negative potentials. When their energy are high enough, electrons are able to transfer into vacant electronic states, i.e. LUMO, of species in the electrolyte, leading to a reduction current. *Vice versa*, imposing a more positive potential decreases the energy of electrons on the working electrode. When the energy is lowered to a certain level, electrons on solutes in the electrolyte will transfer to the working electrode due to a more favourable energy offered there, which results in an oxidation current, as depicted in Figure 2-1.

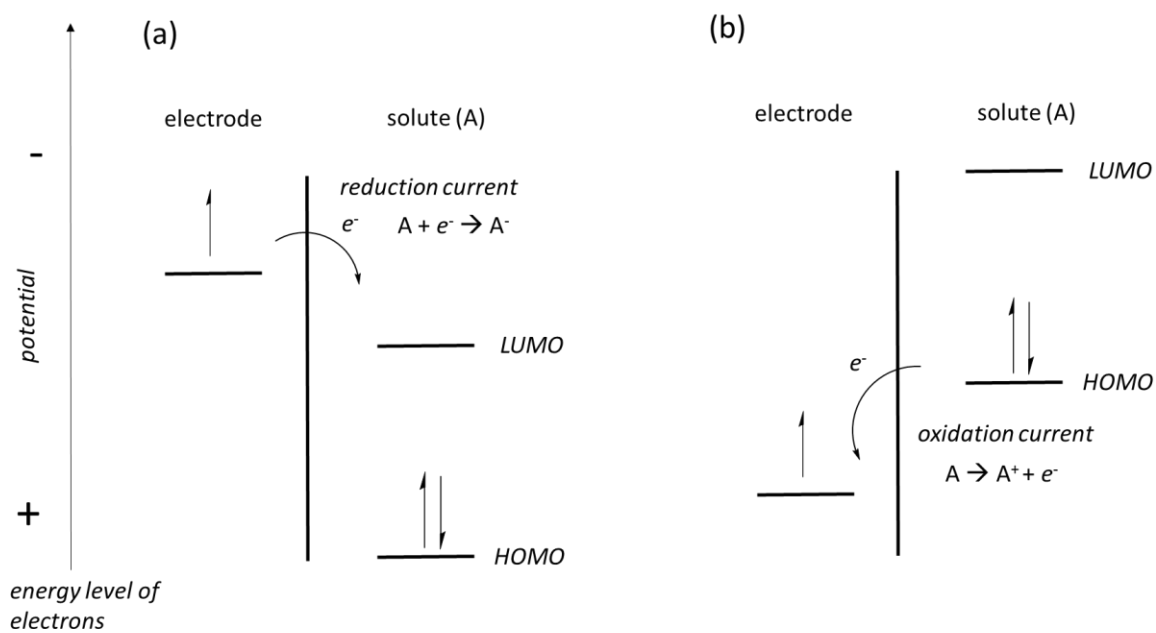


Figure 2-1. A schematic representation of the (a) reduction and (b) oxidation process of solute A with a working electrode in an electrolyte. The molecular orbitals (MO) of species A shown are the highest occupied MO (HOMO) and the lowest unoccupied MO (LUMO).

2.1.2 Faradaic and non-faradaic processes

When a potential is applied to the electrochemical cell, there are two types of processes occurring at the working electrode, faradaic and non-faradaic processes. As mentioned in 2.1.1, the oxidation/reduction current results from electrons being transferred across the electrode-electrolyte interface. Whereby, the amount of substance produced at the electrode is directly proportional to the quantity of charge flowing through the cell governed by Faraday's law, namely faradaic processes. For a non-faradaic process, no charge-transfer reactions occur at the electrode-solution interface. In this process, an applied potential leads to an accumulation of opposite charges on the electrode and the solution side respectively, forming an electrical double layer. Therefore, the electrode-solution interface acts as a capacitor, as shown in Figure 2-2. When the imposed potential changes, the accumulation of charges rearranges, causing an external current to flow. Such an external current is called charging current or capacitance current.

The faradaic processes are of sole interest in the investigation of hydrogenase chemistry throughout this Thesis.

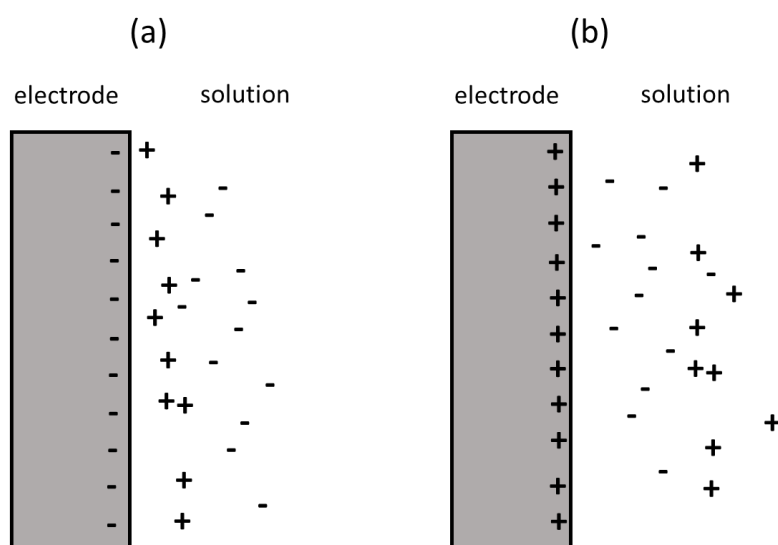


Figure 2-2. Schematic representations of an electrical double layer at the electrode-solution interface. Panel (a): negative charges on the electrode. Panel (b): positive charges on the electrode.

2.1.3 Nernst equation

A redox reaction is composed of two half reactions, *i.e.* oxidation and reduction, as shown in equations below:

An overall reaction of a redox couple, A and B:



Each half reaction with n electrons being involved in can be written as:



A relationship between the equilibrium potential and the concentrations of each species can be expressed by the Nernst equation, as shown in [2-3] and [2-4], where E is the electrode potential; E^0 is the standard redox potential of the redox couple; R is the gas constant; T is the absolute temperature and F is the Faraday constant.

$$E_A = E_A^0 + \frac{RT}{nF} \ln \frac{[A_{ox}]}{[A_{red}]} \dots\dots\dots [2-3]$$

$$E_B = E_B^0 + \frac{RT}{nF} \ln \frac{[B_{ox}]}{[B_{red}]} \dots\dots\dots [2-4]$$

The overall cell potential, E , is the potential difference of the two half reactions.

$$E = E_{A(cathode)} - E_{B(anode)} \dots\dots\dots [2-5]$$

Thus,

$$E = E_{A(cathode)} - E_{B(anode)} = (E_A^0 - E_B^0) + \frac{RT}{nF} \ln \left(\frac{[A_{ox}][B_{red}]}{[A_{red}][B_{ox}]} \right) \dots\dots\dots [2-6]$$

When the applied potential is above the standard redox potential of the reaction, oxidation occurs at the electrode to re-adjust the ratio of the couple in

the electrolyte and electrons flow to the electrode (positive current). Similarly, when the imposed potential is below the standard redox potential of the reaction, a reduction reaction is driven, leading to a flow of electrons from the electrode to the electrolyte (negative current).

On the basis of the relationship expressed in [2-6], a cell potential can be linked to the standard-state free energy of reaction, ΔG^0 , as shown below:

$$\Delta G^0 = -RT \ln K \dots\dots\dots [2-7]$$

$$K = \frac{[A_{red}][B_{ox}]}{[A_{ox}][B_{red}]} \dots\dots\dots [2-8]$$

And thus,

$$E = (E_A^0 - E_B^0) + \frac{1}{nF} \Delta G^0 \dots\dots\dots [2-9]$$

The cell potential is zero when the redox reaction reaches equilibrium, therefore,

$$\Delta G^0 = -nF(E_A^0 - E_B^0) \dots\dots\dots [2-10]$$

From a thermodynamic point of view, a reaction is spontaneous if the standard redox potential of the overall reaction is greater than zero.

2.1.4 2H⁺/H₂ cycling

For a hydrogen production reaction



the Nernst equation is given by equation [2-12],

$$E = E^0 - \frac{2.303RT}{nF} \log\left\{\frac{\rho(H_2)}{(\alpha_{H^+})^2}\right\} \dots\dots\dots [2-12]$$

where α_{H^+} is the activity of H⁺; $\rho(H_2)$ is the H₂ partial pressure; n is number of electrons involved in the redox reaction ($n = 2$ for the interconversion of 2H⁺/H₂); F is the Faraday constant; R is the gas constant and T is the absolute temperature. Thus, RT/F is 26 mV at room temperature and provided that the activity coefficient of H⁺ is 1, the relationship of $E_{(2H^+/H_2)}$ can be re-written to [2-13], where the potentials are in mV.

$$E = E^0 - 29.939(\log(\rho_{H_2}) + 2\text{pH}) \dots\dots\dots [2-13]$$

Under conditions at pH 0; 25 °C; and 1 bar H₂, the standard redox potential of this reaction, E^0 , is 0. It is clear to see that $E_{(2H^+/H_2)}$ is strongly dependent on the partial pressure of H₂ and the concentration of H⁺ (pH); it decreases as pH increases at a rate of -59.8 mV per pH unit and shifts by -29.9 mV per order of magnitude increase in H₂ pressure.

2.1.5 Overpotential

In electrochemistry, overpotential is the potential difference between the potential at which the redox reaction actually happens and a thermodynamically determined potential of the redox reaction under a certain condition. A larger overpotential means more energy is required to drive the reaction in an electrolytic cell.

2.1.6 Protein film electrochemistry

In protein film electrochemistry (PFE), the redox enzymes are immobilised on the working electrode. Thus, the electrode acts as a direct redox partner of the studied enzyme, providing a continuous flow of electrons, back and forth, as the potential is varied.

PFE is one of the techniques exploited in this Thesis for studies of [NiFe]-hydrogenases. Figure 2-3 depicts the configuration of a [NiFe]-hydrogenase molecule in a PFE experiment. An efficient/fast electron flow between the electrode and the enzyme molecule can be achieved if the [NiFe]-hydrogenase molecule is immobilised on the electrode surface in the right orientation, i.e. with the electron entry site, the distal cluster, facing towards the electrode side. Thus, during experiments, provided that mass transport of the substrates does not limit the reaction, namely the substrate is constantly supplied to the electrode and the product is constantly removed to keep the concentration at the electrode surface close to the level in the bulk solution, the faradaic

component of the recorded current is proportional to the catalytic turnover rate of the enzyme.

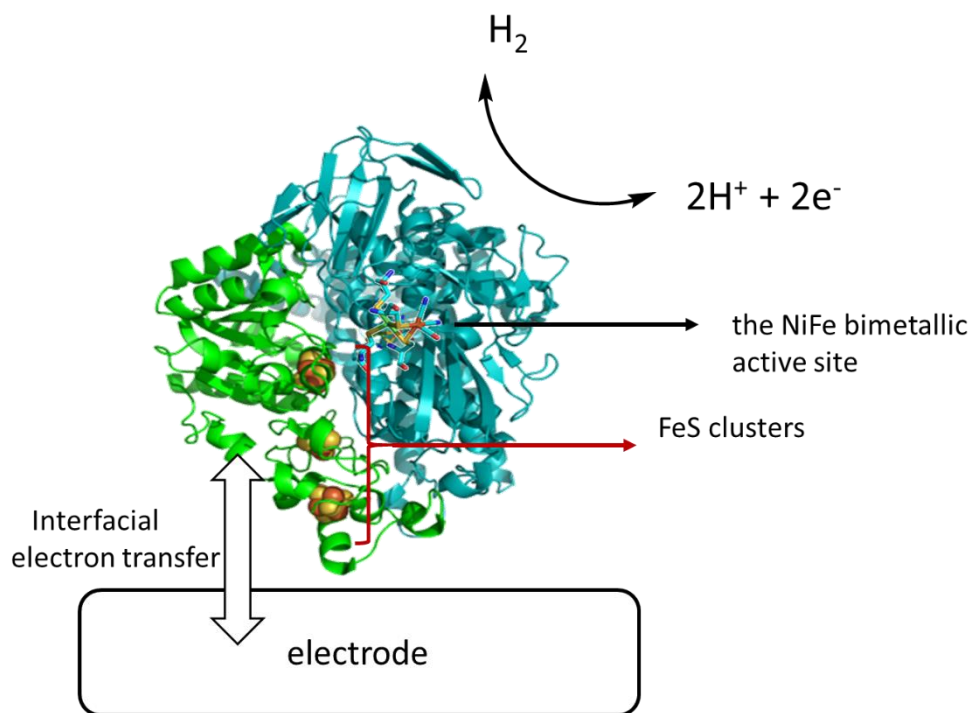


Figure 2-3. A cartoon representative configuration of a [NiFe]-hydrogenase molecule in protein film electrochemistry. Effective electron exchanges between the immobilised enzyme and the electrode can be achieved if the distal FeS cluster (with respect to the bimetallic centre) of the electron relay chain faces towards the electrode side. The FeS clusters are shown as spheres.

The use of a hydrodynamic rotating disk electrode (RDE) in this Thesis ensures the enzymatic catalysis is not limited by mass transport. For a diffusion control process, the current (i) at a planar RDE is given by the Levich equation:

$$i_{(\omega)} = 0.62nFAD^{2/3}\nu^{-1/6}\omega^{1/2}C \dots\dots\dots [2-14]$$

where A is the electrode surface area; D is the diffusion coefficient of the substrate; ν is the kinematic viscosity of the solution; ω is the electrode rotation rate; C is the concentration of the substrate, and other terms have already been defined. Thus, the current is proportional to the square root of electrode rotation speed. However, such a relationship breaks down at the limit when the mass transport control is removed. The observed relationship between current and rotation rate is then given by the Koutecky-Levich equation:

$$\frac{1}{i_{\text{lim}(\omega)}} = \frac{1}{i_{\text{lim}}} + \frac{1}{0.62nFAD^{2/3}\nu^{-1/6}\omega^{1/2}C} \dots\dots\dots [2-15]$$

where $i_{\text{lim}(\omega)}$ is the limiting (plateau) current at rotation rate ω ; i_{lim} is the maximum limiting current achieved at infinite rotation rate; and the other terms have been defined earlier. In theory, i_{lim} is obtained by extrapolation in a plot of $1/i_{\text{lim}(\omega)}$ against $1/\omega^{1/2}$. But in practice, an approximation can be obtained at an electrode rotation rate above which the current and waveform no longer change significantly when ω is increased.

Two main types of measurements are exploited in PFE to unveil hydrogenase chemistry: cyclic voltammetry (CV) and chronoamperometry. In cyclic voltammetry, the electrode potential is swept between two limiting values and the resulting current is monitored simultaneously. This method offers an

overall picture of the catalytic activity of the enzyme, and allows us to identify potentials at which there is an irregularity in catalytic current, due to a transition between active and inactive states, or an existence of an optimal operating potential for the enzymatic catalysis. Figure 2-4 depicts the results of *E. coli* Hyd-2 in a PFE experiment for demonstration. Panel (a) shows a non-faradaic current response (charging current) of a pyrolytic graphite edge (PGE) electrode used as a background in cyclic voltammetry in PFE (recorded prior to the experiments). Panel (b) depicts a typical cyclic voltammogram of *E. coli* Hyd-2 on a PGE electrode recorded at pH 6 under 1 bar H_2 at 1 mV s^{-1} scan rate. The positive current is led by the reaction of H_2 oxidation, whereas the negative current is resulted from the reaction of H^+ reduction. The slight decrease of oxidation current above 0 V is attributed to the formation of an inactive state, Ni-B. The activity is restored on the reverse scan when the potential is swept to values lower than -0.05 V. A characteristic quantity, termed E_{switch} , is defined as the potential of maximum slope in the reductive activation direction, determined from a first derivative plot. E_{switch} is a robust parameter to which can be attached thermodynamic significance; it has been used to compare different potential-dependent inactive states and the ease with which they can be reactivated in various hydrogenases.^{1,75,87}

The second method is chronoamperometry, in which the current is recorded as a function of time. Any changes of catalytic activity by a potential step or introduction of an inhibitor is observed immediately in the current-time trace, whereby useful kinetics information can be extracted (see Figure 2-5). In

Chapter 7, the reaction of *E. coli* Hyd-1 and Hyd-2 with isocyanides were investigated by chronoamperometry for inhibition constant determinations. The theory and methods of this study will be described in more detail in section 2.3.

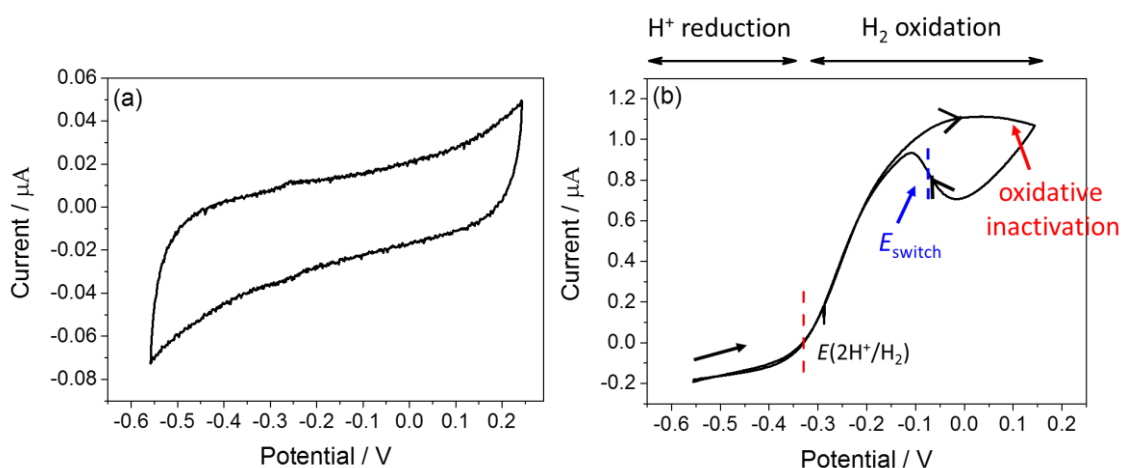


Figure 2-4. (a) A typical current against potential plot, showing a non-faradaic current response (charging current) of a pyrolytic graphite edge (PGE) electrode in cyclic voltammetry for PFE experiments. (b) A cyclic voltammogram of *E. coli* Hyd-2 on a PGE electrode. Experimental conditions: mixed buffer (pH 6); 28 °C; $\omega = 1500$ rpm; scan rate = 1 mV s^{-1} ; 1 bar H_2 . The scan direction is shown by the black arrowheads.

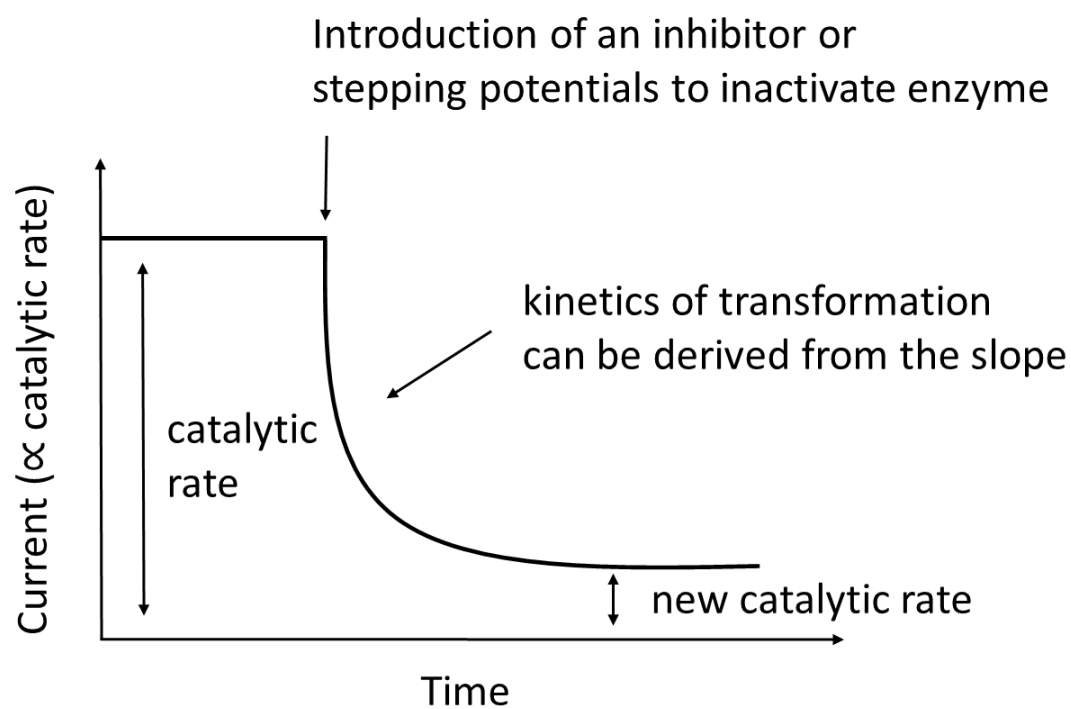


Figure 2-5. A schematic representation of the current-time trace in the chronoamperometry experiment, adapted with permission from reference 1, Copyright © 2007, American Chemical Society.

2.2 Electrochemical methods and apparatus

2.2.1 The three-electrode system

The three electrode system is used in all electrochemical experiments throughout this Thesis, as shown in Figure 2-6. During each experiment, a potential is applied between the working electrode and the reference electrode which has a well-defined potential. A precise potential control can be obtained if the current passing through the reference electrode is minimised; this is achieved by introducing a counter electrode to create a closed loop. In this set-up, the interfacial potential difference at the reference electrode remains fixed, contributed by the constant species composition on the electrode surface.

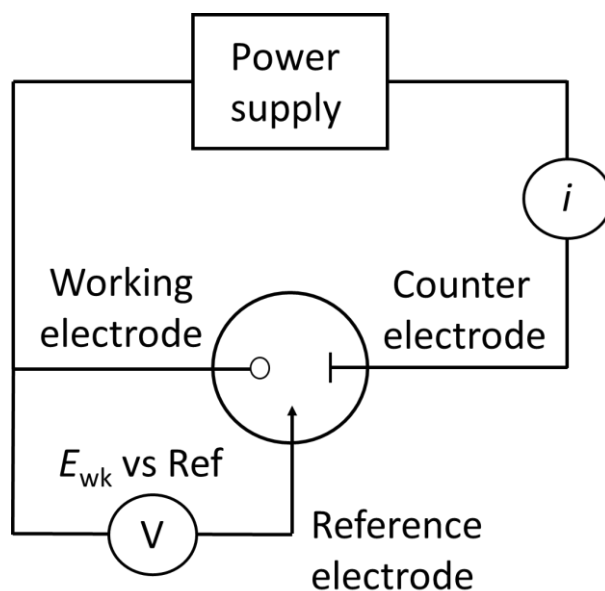


Figure 2-6. A schematic circuit diagram of a three electrode system.

2.2.2 The Electrochemical apparatus

A homemade glass electrochemical cell is used for PFE experiments, as shown in Figure 2-7. The temperature during experiments is controlled by a water jacket, which is fitted into the cell and connected to a water circulator (Grant, GD120). Platinum wire (Surepure Chemicals) is used as a counter electrode, and a saturated calomel electrode (SCE, BASi) is used as a reference electrode. A hydrodynamic working electrode, rotating disk electrode (RDE), is connected to the electrode rotator (EG&G model 636) for efficient substrate supply and product removal. Below the counter electrode compartment, there is a special designed sidearm, which allows the injection of inhibitors during experiments. Experiments are carried out using a potentiostat (Autolab PGSTAT 128N, EcoChemie, Netherlands) manipulated by GPES software (EcoChemie). Gas flow controllers (Brooks Instrument) are exploited to provide accurate mixtures of gases.

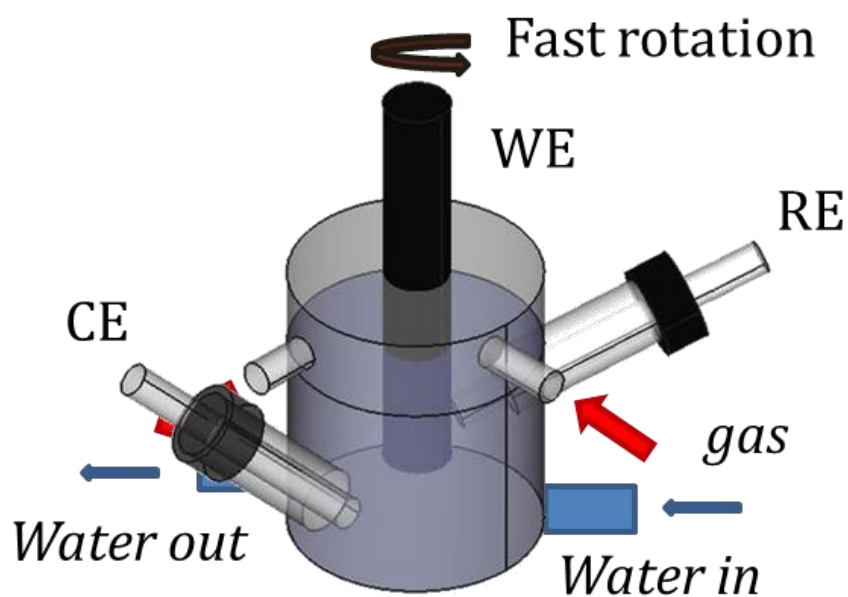


Figure 2-7. A schematic diagram of the apparatus used for protein film electrochemistry experiments. Accurate mixtures of gases are obtained by using gas flow controllers.

2.2.3 Preparation of an electroactive film of hydrogenases for PFE

The working electrode used for hydrogenase studies in PFE in this Thesis is a pyrolytic graphite edge (PGE) electrode (Momentive Performance Materials), which is built by incorporating a PGE rod (2 mm diameter) into a Delrin rotating disk electrode case; the planar surface area of the electrode is 0.03 cm². The graphite rod has a special orientation with edges of the well-ordered layers of graphene sheets facing towards the electrolyte (See Figure 2-8).

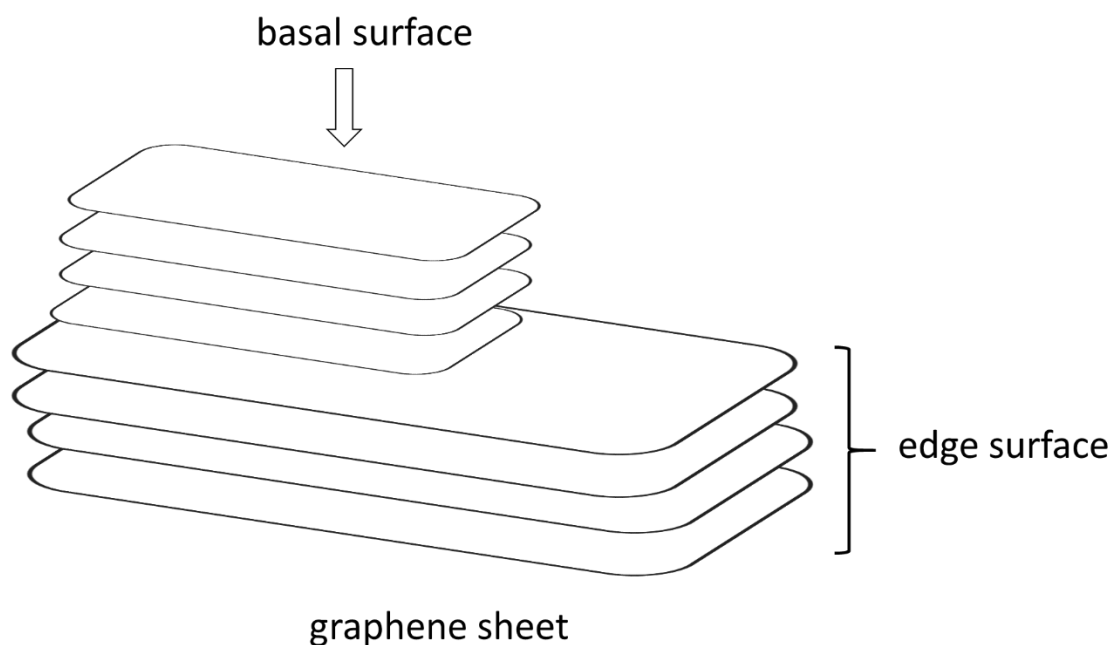


Figure 2-8. A cartoon representation of pyrolytic graphite showing orientations of the edge (PGE) and basal (PGB) surfaces.

A straightforward way to make an electroactive enzyme film is a drop-casting method, in which the enzyme molecules are immobilised on the electrode surface via physical adsorption. A typical procedure of film preparation for

E. coli Hyd-1 and Hyd-2 is carried out as follows: the PGE electrode was freshly abraded with P800 sandpaper or aqueous slurry of 1 μm α -alumina (Buehler Micropolish), before being subject to sonication for approximately 10 s in Milli-Q water, and then rinsed with purified water. A 0.5 μL aliquot of dilute hydrogenase solution (*ca.* 10 μM) was then pipetted onto the electrode surface, and left for approximately 15 s for adsorption before rinsing the electrode to remove the unadsorbed enzyme. The electrode was then immersed in enzyme-free buffer solution in equilibrium with H_2 for electrochemical measurements. This method works very well for enzyme adsorption,¹ and the reasons are as follows: (i) there are a range of oxidised carbon functionalities (such as $\text{C}=\text{O}$, $\text{C}-\text{OH}$ and COOH) on the PGE surface;⁸⁸ and (ii) abrading the electrode with sandpaper makes the PGE surface extremely rough with deep cracks and defects, as shown in the scanning electron microscope (SEM) image adopted from reference ⁸⁸ (Figure 2-9). These factors are regarded as important roles in providing adsorption sites for a diverse range of proteins with different surface characteristics.

Moreover, a carbon electrode provides a protein-compatible environment for enzyme adsorption and is chemically inert. It is suitable for operation in a wide potential range for electrochemical experiments on redox proteins (approximately -1 to +1 V vs SHE) , compared with metal electrodes, such as gold and silver electrodes, which are usually protected with the alkanethiol species to avoid denaturing proteins, but proton reduction and desorption of

the thiol layer occur below *ca.* -400 and -300 mV for gold and silver electrodes, respectively.¹⁸

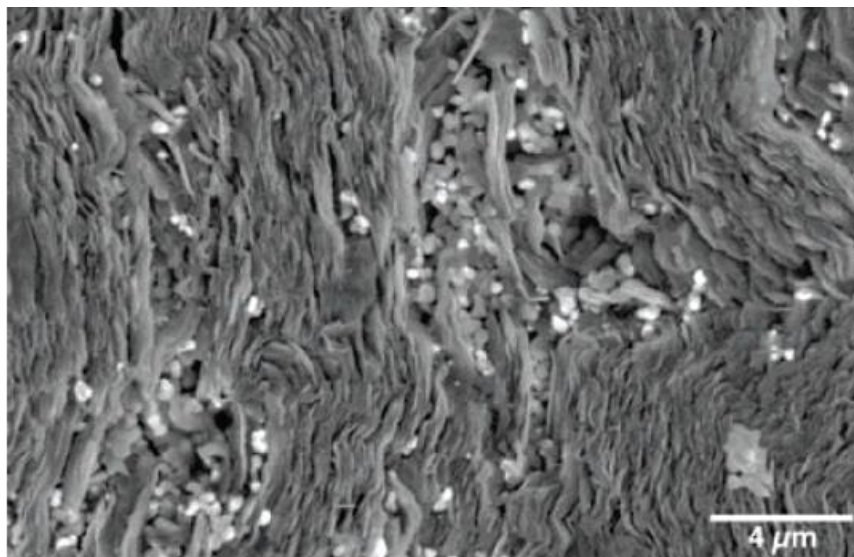


Figure 2-9. A scanning electron micrograph of the “edge” surface of pyrolytic graphite (polished with aqueous slurry of 1 μm α -alumina) with particles of alumina retained on the surface, which can be removed upon further sonication. The figure is adopted with permission from reference ⁸⁸, Copyright © 2006, Springer-Verlag.

2.2.4 Reference potentials

In electrochemistry experiments, potentials are often measured and quoted with respect to the standard hydrogen electrode (SHE). It is defined by the simplest half-reaction, the reduction of protons to dihydrogen occurring at a Pt electrode, in an aqueous solution at pH 0, 25 °C, in equilibrium with 1 bar H₂ to keep all components at unity activity.

However, in practice, it is hard to use the SHE in experiments due to cumbersome apparatus and the need to maintain H₂ at 1 bar pressure. Alternatively, other electrodes with well-defined potential difference against the SHE are exploited experimentally, and then the potentials are converted to standard values in data presentations. For all experiments described in this Thesis, the saturated calomel reference electrode (SCE) is used to provide potential control, which is composed of a mixture of mercury and mercury chloride in contact with saturated potassium chloride solution. Potentials (E , in volts, V) are then quoted relative to the SHE, using the conversion $E_{\text{SHE}} = E_{\text{SCE}} + 0.242 \text{ V}$ at 25 °C.⁸⁹

2.3 Enzyme kinetics and inhibition

2.3.1 Michaelis-Menten kinetics⁹⁰

A simple model proposed by Leonor Michaelis and Maud Leonora Menten to describe the reaction occurring between an enzyme molecule and its substrate is depicted in [2-16], which has been widely used in studies of enzyme kinetics.



The idea of this model is: when the enzyme molecule, E , reacts with the substrate, S , an enzyme-substrate complex, ES , forms with a rate constant of k_1 . The complex, ES , can either proceed to form the product, P , with a rate constant of k_2 , or dissociates to re-form the original species, E and S , with a rate constant of k_{-1} . Provided that product formation is the rate-determining step ($k_2 \sim < k_1$ and k_{-1}), the catalytic rate (v) can be simplified to be solely dependent on the concentration of ES complex and k_2 , as expressed in [2-17].

$$v = k_2[ES] \quad \dots\dots\dots [2-17]$$

Also, since k_2 is much smaller than k_1 and k_{-1} , the concentration of the intermediate, $[ES]$, is almost the same throughout the reaction, which is the so-called steady-state approximation. Applying this approximation to [2-16], which means the rate of ES formation is equal to the rate of its consumption, [2-18] is derived:

$$k_1[E][S] = (k_{-1} + k_2)[ES] \quad \dots\dots\dots [2-18]$$

Rearranging [2-18] gives a new constant, called the Michaelis constant, K_M (2-19).

$$K_M = \frac{[E][S]}{[ES]} = \frac{k_{-1}+k_2}{k_1} \dots\dots\dots [2-19]$$

K_M is a measure of the affinity of an enzyme for its substrate; a high K_M value indicates a low affinity.

The total enzyme concentration, $[E]_T$, throughout the reaction is equal to the sum of concentrations of free enzyme, $[E]$, and the enzyme-substrate complex, $[ES]$, as shown in [2-20].

$$[E]_T = [E] + [ES] \dots\dots\dots [2-20]$$

Thus, the catalytic rate can be written in [2-21]:

$$v = k_2[E]_T \left(\frac{[S]}{K_M + [S]} \right) \dots\dots\dots [2-21]$$

A maximal catalytic rate (V_{max}) is achieved at an extremely high concentration of the substrate, which saturates the catalytic site of the enzyme:

$$V_{max} = k_2[E]_T \dots\dots\dots [2-22]$$

Substituting equation [2-22] into [2-21] yields the Michaelis-Menten rate equation:

$$v = V_{max} \left(\frac{[S]}{K_M + [S]} \right) \dots\dots\dots [2-23]$$

A plot of v against $[S]$ gives a hyperbolic curve passing through the origin as depicted in Figure 2-10. According to equation [2-23], it is clear to see that when $[S] = K_M$, the Michaelis-Menten equation gives $v = \frac{1}{2} V_{max}$. Therefore, K_M not only serves as an indicator to reveal the affinity between an enzyme and its

substrate, but also is the substrate concentration at which half the limiting rate is obtained.

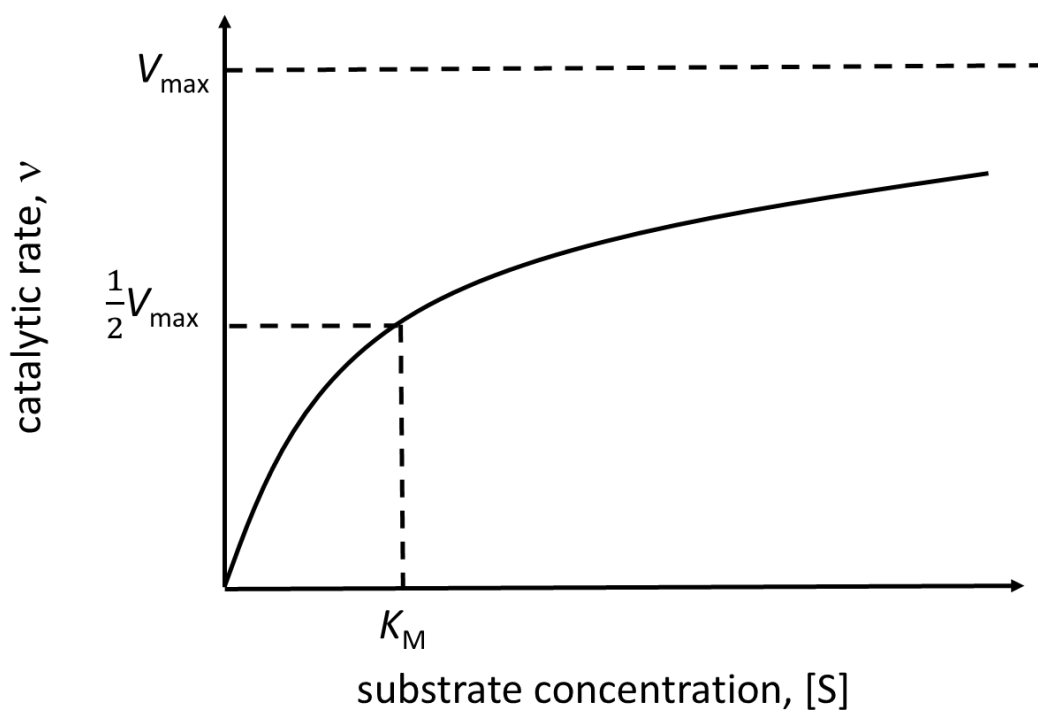


Figure 2-10. A plot of enzyme reaction rate, v , against substrate concentration, $[S]$, according to the Michaelis-Menten equation.

2.3.2 Types of inhibition

Inhibitors are substances which decrease the catalytic activities of enzymes. During a normal enzymatic catalysis, the substrate binds to the active site of a free enzyme quickly, forming an active enzyme-substrate complex, ES . Such a complex either proceeds to generate a product, or dissociates to re-form an active enzyme (Figure 2-11, panel a). When an inhibitor (I) is present, there are three different ways for it to block the activity of an enzyme: (i) competitive inhibition, the inhibitor binds to the same place in the active site where the substrate coordinates to reversibly, and has a stronger affinity towards the active enzyme than the substrate. Therefore, an enzyme-inhibitor complex (EI) forms predominantly (Figure 2-11, panel b); (ii) uncompetitive inhibition, an inhibitor binds to the enzyme-substrate complex, forming EIS complex and prohibiting the following product formation (Figure 2-11, panel c); and (iii) mixed type inhibition, an inhibitor prevents the catalysis by binding to both of the free enzyme and the enzyme-substrate complex.

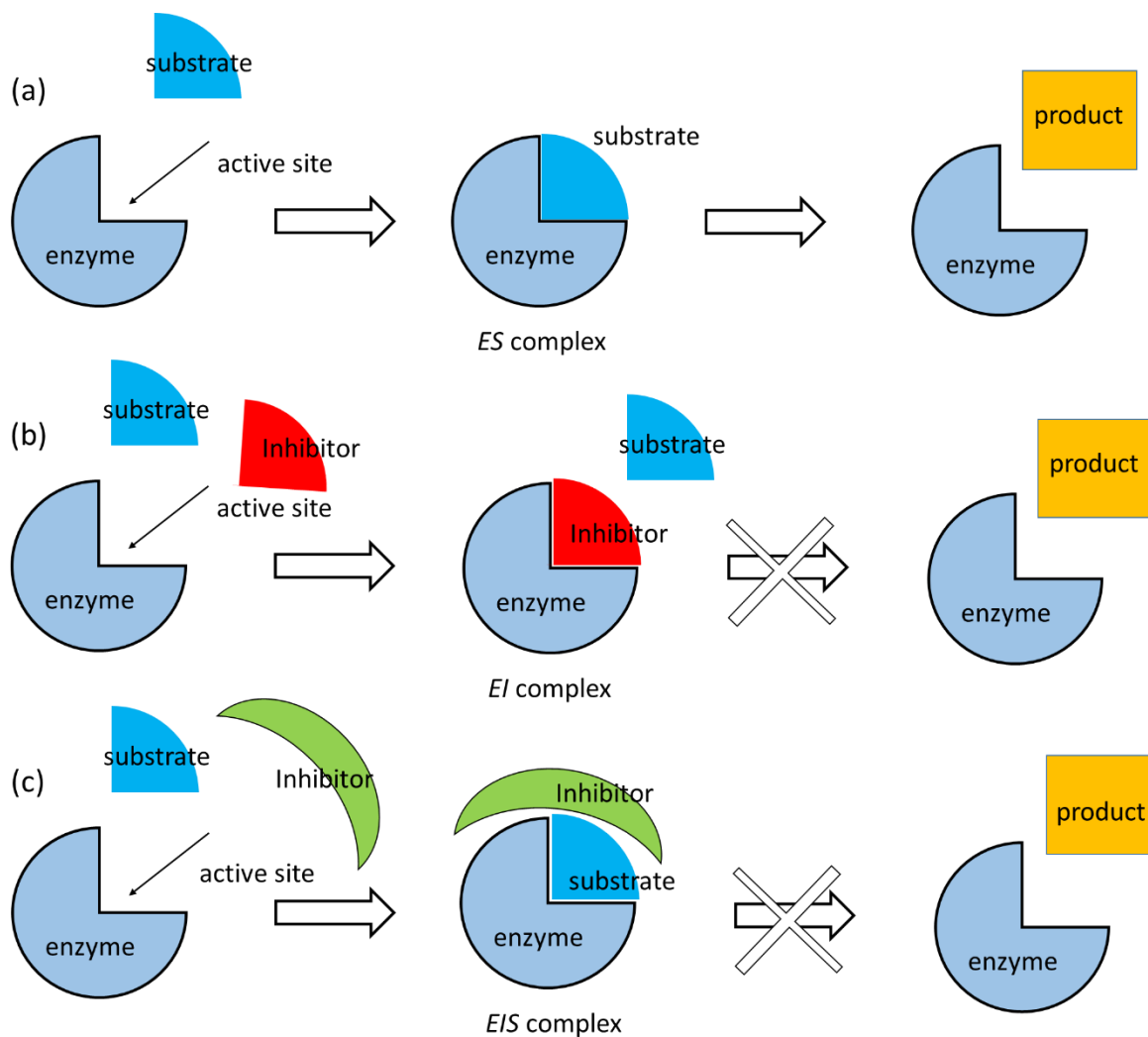


Figure 2-11. Cartoon representations of (a) typical enzymatic catalysis with the substrate; (b) competitive inhibition; and (c) uncompetitive inhibition.

2.3.3 Ways of determination of inhibition constants⁹⁰

A quantitative method was developed to determine the inhibition constant for the competitive reaction by a modified Michaelis-Menten rate equation (Dixon, 1953, see appendix for detailed derivation)⁹¹, as shown in [2-24].

$$v = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_I}\right) + [S]} \dots\dots\dots [2-24]$$

where K_I is the dissociation constant of the EI complex, or termed inhibition constant, for the competitive inhibition; a high K_I value indicates a weak inhibition. Other terms have already been defined before. Taking reciprocals of both sides of [2-24] gives equation [2-25]:

$$\frac{1}{v} = \frac{K_M[I]}{V_{max}[S]K_I} + \frac{1}{V_{max}} \left(1 + \frac{K_M}{[S]}\right) \dots\dots\dots [2-25]$$

A plot of $1/v$ against the inhibitor concentration of $[I]$, at a given substrate concentration of $[S]$, gives a straight line (which is the so-called Dixon plot). Carrying out the experiments at various substrate concentrations generates a set of lines intersecting at a point where $1/v$, $[I]$ and V_{max} are the same for all lines. The concentration of inhibitor at the intersection point is equal to $-K_I$ by this method (Figure 2-12, panel a).

Cornish-Bowden further showed that the rate equation can be expressed as a general case ([2-26]) by linear combination of all common types of inhibition:⁹²

$$v = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_I}\right) + [S]\left(1 + \frac{[I]}{K_I'}\right)} \dots\dots\dots [2-26]$$

where K_I' represents the dissociation constant of the EIS complex. A Dixon plot of the mixed type reaction using [2-26] gives an intersection point, the concentration of inhibitor at which is equal to $-K_I$ (Figure 2-12, panel c), but the information on K_I' is missing. A set of parallel lines is obtained in the Dixon plot using [2-26] for uncompetitive inhibitions, because the K_I is infinite (Figure 2-12, panel b).

[2-27] is derived following the reciprocation of [2-26] multiplied with [S]:

$$\frac{[S]}{v} = \frac{K_M}{V_{max}} \left(1 + \frac{[I]}{K_I}\right) + \frac{[S]}{V_{max}} \left(1 + \frac{[I]}{K_I'}\right) \dots\dots\dots [2-27]$$

A plot of $[S]/v$ against $[I]$ is termed a Cornish-Bowden plot, which is complementary to a Dixon plot because the K_I' is able to be determined in this graphical method.⁹² Experiments carried out at various $[S]$ give a set of parallel lines for the competitive inhibition, and intersection points for the uncompetitive and mixed type reactions, the concentration of inhibitor at which are equal to $-K_I'$ (Figure 2-12, panel d-f). Since a Dixon plot does not distinguish a competitive reaction from a mixed-type inhibition, and a Cornish-Bowden plot does not always distinguish an uncompetitive reaction from a mixed-type inhibition, a combination of both methods is able to provide complementary information for characterisation.

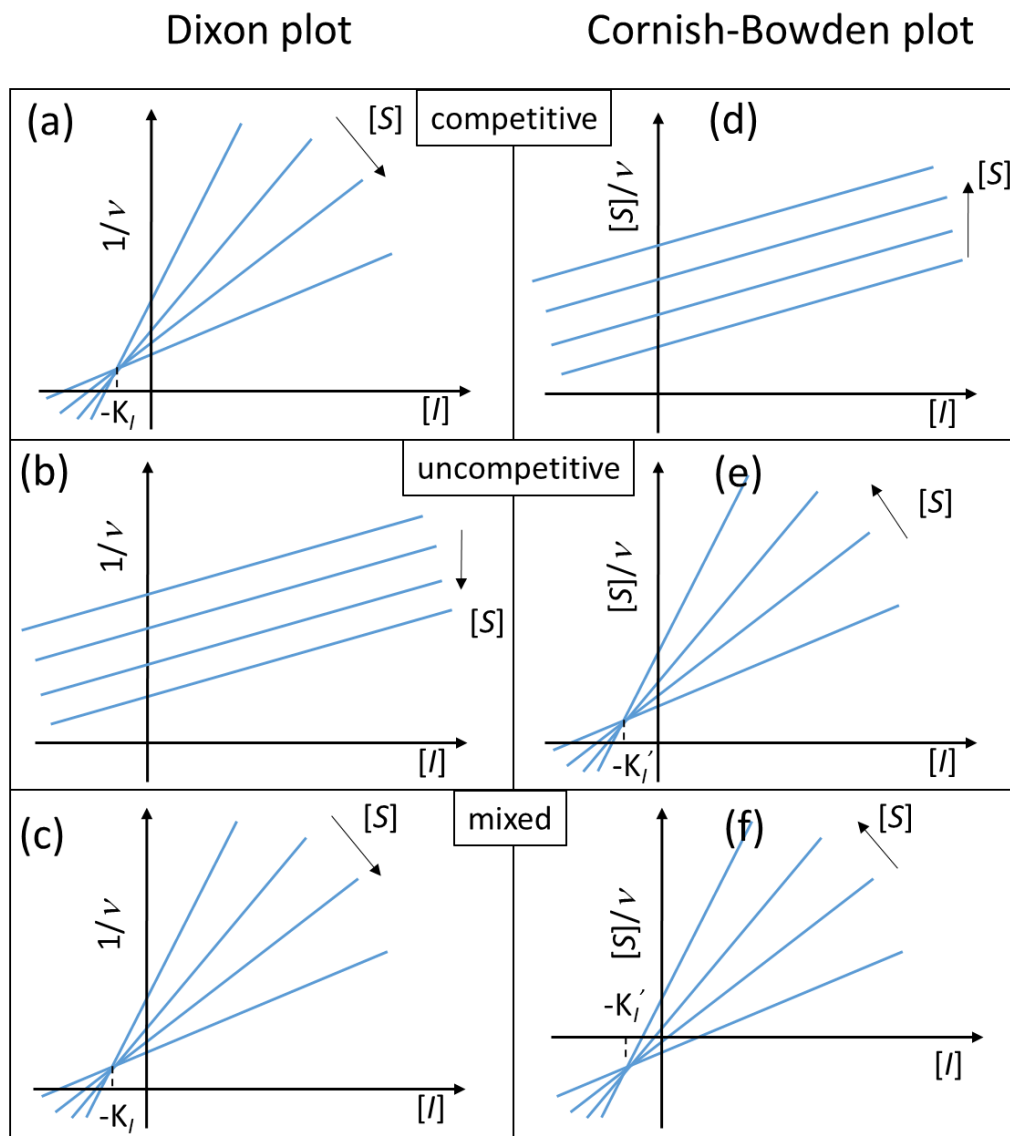


Figure 2-12. Characteristic plots for competitive, uncompetitive and mixed type inhibitions.

2.4 Infrared spectroscopy^{93,94}

2.4.1 Molecular vibrations and selection rules

Infrared spectra result from transitions between quantised vibrational energy states of molecules, which falls in the infrared region of the electromagnetic spectrum. Infrared spectroscopy is commonly carried out in the mid-infrared range (mid-IR, 4000 – 400 cm⁻¹) as it is the region where many functional groups give rise to characteristic absorptions. Molecular vibrations can be just a simple motion of the two atoms in a diatomic molecule or a complex motion with all atoms in a polyfunctional molecule coupled together. For a non-linear molecule containing N atoms, there are 3N-6 ways (the so-called vibrational modes) for atoms to vibrate, whereas a linear molecule has 3N-5 different modes in vibration.

At low energies, namely low values of vibrational quantum number (n , *vide infra*), molecular vibrations can be described approximately by a simple harmonic model. In which, a molecule is represented by a series of point masses (atomic nuclei), which are connected by springs (chemical bonds). For each vibrational mode, the movement of atoms obeys Hooke's Law [2-28]: the restoring force, F , is proportional to the nuclear displacement, r , linked by a force constant, k .

$$F = -kr \text{ [2-28]}$$

Which gives a parabolic potential energy surface as a function of the displacement of the atoms from their equilibrium positions (Figure 2-13, solid

line). The quantised vibrational energy of the i th mode, V_i , is described as [2-29]:

$$V_i = h\nu_i(n_i + \frac{1}{2}) \dots\dots\dots [2-29]$$

where h is Planck's constant, ν_i is the fundamental frequency of the particular mode, and n_i is the vibrational quantum number of the i th mode ($n=0, 1, 2, 3\dots$).

If the vibrational modes were strictly harmonic, only transitions involving changes in n (Δn_i) equal to ± 1 would be allowed. However, in reality, especially when n is large, V_i must be expressed by an anharmonic (Morse-type) potential function (Figure 2-13, dashed lines). The potential energy is given to a first approximation by [2-30]:

$$V_i = h\nu_i(n_i + \frac{1}{2}) + h\nu_i\chi_i(n_i + \frac{1}{2})^2 \dots\dots\dots [2-30]$$

where χ_i is the anharmonicity constant, which is dimensionless and typically has values between -0.001 and -0.02, depending on the mode. The effect of anharmonicity relaxes the selection rule to let transition with $|\Delta n_i| > 1$ be allowed.

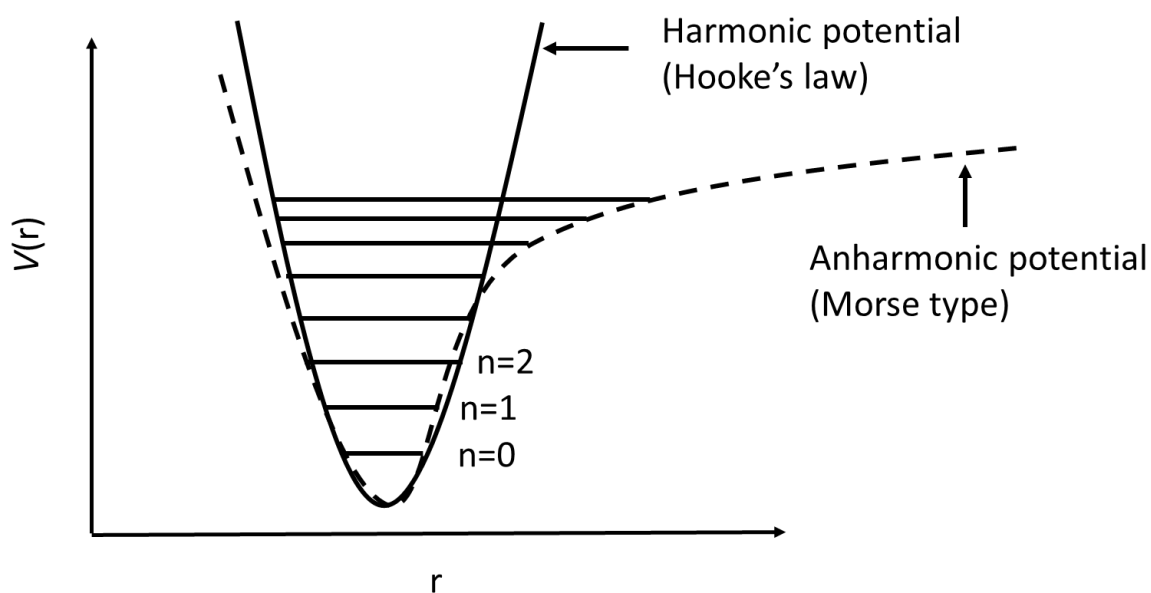


Figure 2-13. Potential energy, $V(r)$, of a diatomic molecule as a function of the atomic displacement, r , from the equilibrium position during a vibration for a harmonic oscillator (solid line) and an anharmonic oscillator (dashed lines).

2.4.2 Beer-Lambert Law

The amount of light radiation absorbed by a substance can be related to the thickness and the concentration of the sample as follows:

$$A = \varepsilon cl \dots\dots\dots [2-31]$$

where A is the absorbance of the substance; c is the concentration and l is the thickness of the sample; ε is the constant of proportionality, which is referred to as the molar absorptivity.

The absorbance is equal to the difference between the logarithms of the intensity of the light entering the sample (I_0) and the intensity of the light transmitted (I) by the sample, and therefore it is dimensionless.

$$A = \log I_0 - \log I = \log\left(\frac{I_0}{I}\right) \dots\dots\dots [2-32]$$

Another parameter, termed transmittance (T), is defined as the ratio of the intensity of the light emitted by the sample over the intensity of the light received by the sample:

$$T = \frac{I}{I_0} \dots\dots\dots [2-33]$$

Therefore,

$$T = 10^{-A} \dots\dots\dots [2-34]$$

A term of optical density (O.D.) is normally used as the unit to describe absorbance in practice.

2.4.3 Force constants and electronic effect

On the basis of Hooke's Law [2-28], the fundamental frequency of a chemical bond of a particular mode is expressed as [2-35]:

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \dots\dots\dots [2-35]$$

where μ is the reduced mass of two nuclei with masses m_1 and m_2 :

$$\mu = \frac{m_1 m_2}{m_1 + m_2} \dots\dots\dots [2-36]$$

Thus, vibrational frequencies in infrared spectroscopy report directly on the force constant (k) of a bond. This feature serves as a good probe of electronic behaviour in organometallic complexes, especially for which the metals are coordinated with ligands having strong absorption of infrared radiation, such as CO and CN $\cdot$ (the hydrogenase active site in this Thesis).

How electronic characteristics of ligands are affected by interaction with the metal in an organometallic complex can be explained by the Chatt-Dewar-Duncanson model;⁹⁵ CO is used for demonstration, as shown in Figure 2-14. Panel a depicts the molecular orbital (MO) diagram of CO involved in bond formation with a metal. The two degenerate lowest unoccupied molecular orbitals (LUMOs) can accept electron densities via forming π -bonds with the metal d orbitals of compatible symmetry and energy (panel b), and this is why CO is termed a π -acid ligand. Whereas, the highest occupied molecular orbital (HOMO) has a σ feature, and can donate electron densities to empty metal orbitals of appropriate symmetry and energy via a σ -bond (panel c). Moreover, an increase of electron density of the metal imposed by other ligands can

redistribute to the LUMOs of the coordinated CO ligand by a synergic process, which is the so-called “back-donation”.

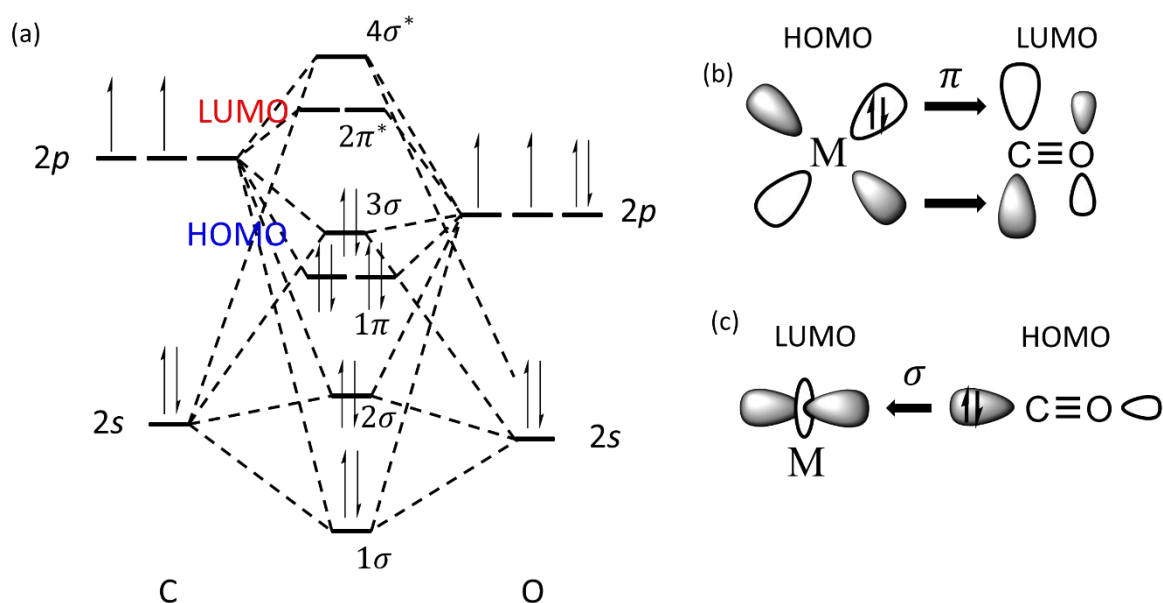


Figure 2-14. Panel a: Molecular orbital (MO) diagram of CO, highlighting the HOMO (3 σ) and LUMO (2 π^*). Panel b and c: cartoon representations of Chatt-Dewar-Duncanson model for description of bonding between a metal (M) and a diatomic ligand (CO here) with interactions between Highest Occupied Molecular Orbitals (HOMOs) and Lowest Unoccupied Molecular Orbitals (LUMOs) of compatible energy and symmetry.

The bond strength of CO in organometallic complexes is affected significantly by these two types of interactions mentioned above. The HOMO has a weakly antibonding character, and therefore a donation of electron density from this orbital gives a net effect to strengthen the CO bond. Thus, the infrared absorption band, ν_{CO} , is upward to higher wavenumber. The back donation of electron density from the metal to the strongly antibonding LUMOs weakens the CO bond, leading to a downward shift of ν_{CO} . Such a feature is very sensitive to the formal oxidation state of the metal, as demonstrated by a series of iron carbonyl species (Table 2-1).⁹⁶ ν_{CO} of gaseous carbon monoxide is centred at 2143 cm^{-1} .⁹⁷ In the complex $[\text{Fe}(\text{CO})_6]^{2+}$ (the formal oxidation state of Fe is II), the interaction of CO with metal is more weighted toward σ -donation, and thus the CO bond is strengthened to have a higher vibrational energy, 2215 cm^{-1} . The interaction is more dominated by π -acceptance as the electron density of the metal increases (i.e. a decrease in its formal oxidation state), which leads to a downward shift in ν_{CO} .

Table 2-1. ν_{CO} of three iron carbonyl complexes observed in infrared spectroscopy.⁹⁶

	$[\text{Fe}(\text{CO})_6]^{2+}$	$\text{Fe}(\text{CO})_5$	$[\text{Fe}(\text{CO})_4]^{2-}$
formal oxidation state of iron	+II	0	-II
$\nu_{\text{CO}} / \text{cm}^{-1}$	2215	2030	1815

As for another common π -acid ligand, cyanide (CN^-), it is isoelectronic with CO, and has a comparable MO arrangement to coordinate to a metal through carbon. However, its negatively charged property makes it as a better σ donor but a poorer π acceptor ligand compared with CO.

[NiFe]-hydrogenases have the built-in π -acid ligands, CO and CN^- , coordinating to the Fe of the active site (Chapter 1, Figure 1-3). Although the Fe stays as low spin Fe^{II} throughout the spectroscopically observed enzyme states, these π -acid ligands still sense the changes in oxidation states of the neighbouring nickel atom (alternation between III, II and I), and the coordination environment of the active site (see section 4.1 for more details). This is contributed by some degree of orbital overlapping between these two metals. Thus, these π -acid ligands serve as a good diagnostic tool in infrared spectroscopy to study hydrogenase chemistry. The vibrational frequencies of these π -acid ligands are well studied and reported for a range of hydrogenases (Chapter 4, Table 4-1); ν_{CO} ranges from 1965 -1860 cm^{-1} , whilst ν_{CN} is observed between 2105 – 2025 cm^{-1} among different redox states.⁴⁶

2.4.4 Instrumentation for infrared spectroscopy

A spectrometer for infrared spectroscopy is designed to measure the amount of infrared radiation absorbed by the sample of interest in a layout shown in Figure 2-15; each component will be described individually.

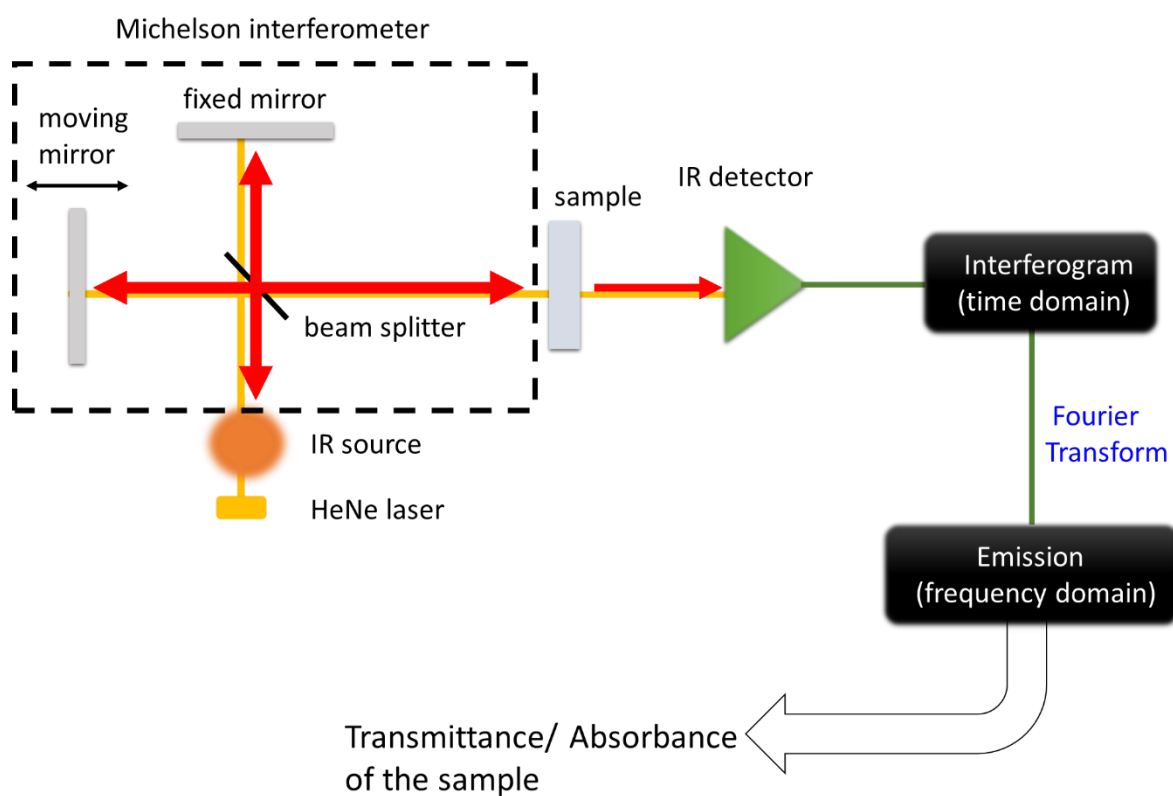


Figure 2-15. Schematic diagram of an infrared spectrometer layout.

Source of infrared radiation

A common source used for infrared spectroscopy is a solid element, such as Globar (a silicon carbide rod) or Nernst glower (a cylindrical rod or tube composed of a mixture of certain oxides, such as ZrO_2 , Y_2O_3 , and Er_2O_3), which can emit steady black-body radiation in the infrared region upon being heated electrically to high temperatures.

In this Thesis, a ceramic element is incorporated into the Varian 680-IR spectrometer as the infrared source, which offers a uniform emissivity over the range $50 - 9600 \text{ cm}^{-1}$; the operation temperatures of this material lie in the range of $1600 - 2400 \text{ K}$.

Detector

A semiconductor (mercury cadmium telluride, MCT) detector is used in experiments. Each infrared photon with sufficient energy, i.e. with energy higher than the band gap of the semiconductor material, moves an electron from the valence band to the conduction band of the semiconductor when it reaches the detector. The generated circuits are further transformed into an electric signal. The mercury cadmium telluride material is contained in a vacuum sealed case. During experiments, the detector has to be cooled with liquid nitrogen to a temperature of 77 K to reduce noise arising from thermal excitations in the material.

Interferometer

A modern spectrometer is normally equipped with an interferometer for efficient data acquisition. In conventional dispersive infrared spectrometers, monochromators are exploited to limit the wavelength range of the radiation reaching the detector to a certain resolution width. A longer time is required in such a set-up to collect a full scan of a spectrum with a given range, such as $4000\text{-}400\text{ cm}^{-1}$, because radiations (with different energy) have to be monitored sequentially. In sharp contrast, a whole spectral range of interest is able to be collected simultaneously by Fourier-transform infrared (FTIR) spectrometer, in which an interferometer replaces the traditional monochromators.

The most common interferometer used in FTIR spectrometers is a Michelson interferometer, which consists of two perpendicular plane mirrors (one is fixed, and the other is free to move in a direction perpendicular to the plane), and a semi-reflecting film (termed the beam splitter) in the middle (Figure 2-15). For an ideal beam splitter, 50% of the incident radiation from the light source is transmitted through it to one of the mirrors, while 50% is reflected to the other mirror. The two beams are reflected from these mirrors, returning to the beam splitter where they recombine and interfere. The beam which emerges from the interferometer to the detector is called the transmitted beam, which is at 90° to the input radiation from the infrared source. Adjusting the position of the moving mirror produces optical path length difference between the two beams, resulting in constructive or destructive interference to the transmitted beam. This means the intensity of the radiation monitored by the detector changes as

a function of time, because the velocity of the moving mirror is known. A mathematical algorithm of Fourier transform can be further applied to convert the intensity of the transmitted beam as a function of frequency, generating the final spectrum readily. A HeNe laser is incorporated with the infrared source to produce standard fringes, by which an accurate position of the moving mirror can be determined all the time.

A better signal-to-noise ratio (S/N) is achieved using a FTIR spectrometer than a dispersive spectrometer due to the following reasons: (i) throughput effect, known as Jacquinot's advantage: the total radiation source output in FTIR spectrometer can pass through the sample and the detector continuously, because there is no monochromator which blocks most of the radiation from reaching them; and (ii) multiplex effect, known as Fellgett advantage: a shorter time is taken for a scan of the spectrum by a FTIR spectrometer, so numerous times of measurements can be done in a given time. Therefore, the S/N is improved significantly by signal-averaging (proportional to the square root of the number of co-added scans).

2.5 Protein film infrared electrochemistry (PFIRE)

Spectroelectrochemistry, which couples FTIR spectroscopy and electrochemistry together, is the method used in this Thesis to carry out potential-dependent investigations on hydrogenases. The traditional method of spectroelectrochemistry is to utilise a cocktail of redox mediators for electrochemical control over the enzymes.⁴⁶ However, a new approach is exploited in this Thesis, in which hydrogenases are immobilised on the working electrode (carbon nanoparticles), and thus direct electrochemical control over the enzyme can be achieved, while a FTIR measurement is carried out (by means of ATR-IR technique, *vide infra*). This technique is referred to as protein film infrared electrochemistry (PFIRE) here (Figure 2-16), and potentially provides a valuable opportunity to catch transient intermediates for further studies on the catalytic cycle.

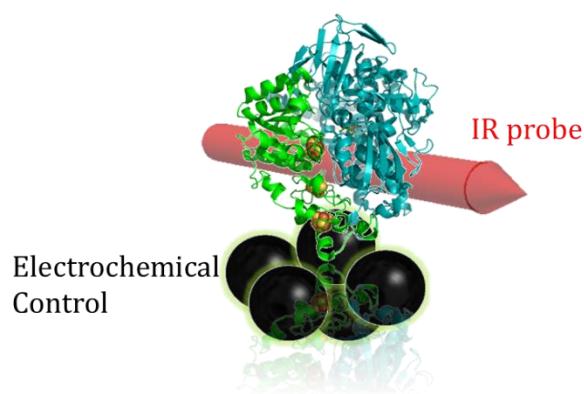


Figure 2-16. Cartoon representation of the concept of direct spectroelectrochemistry. In this technique, enzymes are immobilised on carbon

nanoparticles, and thus direct electrochemical control can be achieved, while a FTIR spectroscopic measurement is conducted (by means of ATR-IR technique).

2.5.1 Attenuated total reflectance (ATR) FTIR

Attenuated total reflectance (ATR) is a sampling method; it is used to study interfacial chemistry in conjunction with various spectroscopic techniques, such as FTIR or Raman spectroscopy. In this Thesis, the configuration of ATR-IR is utilised in PFIRE. Figure 2-17 shows the working principle of this methodology. Total internal reflection occurs when the radiation propagates from optically dense medium with refractive index n_1 (an internal reflection element in this instance), to the medium with lower optical density with refractive index n_2 (aqueous electrolyte solutions in experiments). When the angle of incidence exceeds the critical angle, θ_c (defined in equation [2-37]), an evanescent wave forms and penetrates to the less-dense medium with exponentially-decaying intensity as a function of distance. The depth of penetration (d_p) is defined as the depth at which the original radiation intensity, I_0 , decays to a value of I_0e^{-1} , and can be estimated using equation [2-38].

$$\theta_c = \sin^{-1} \frac{n_2}{n_1} \dots\dots\dots [2-37]$$

$$d_p = \frac{\lambda_0}{2\pi(n_1^2 \sin^2 \theta_1 - n_2^2)^{1/2}} \dots\dots\dots [2-38]$$

Where λ_0 denotes wavelength of the radiation in vacuum and other parameters have been defined above. For experiments in this Thesis, a silicon crystal (with a refractive index of 3.42 at 2000 cm⁻¹) is used as the internal reflection element (8.39×5×1 mm³ with an incidence angle of 39°, Crystal GmbH). A penetration

depth (d_p) at 2000 cm^{-1} is calculated to be $0.47\text{ }\mu\text{m}$ using a refractive index value of 1.33 for aqueous electrolyte medium.⁹⁸

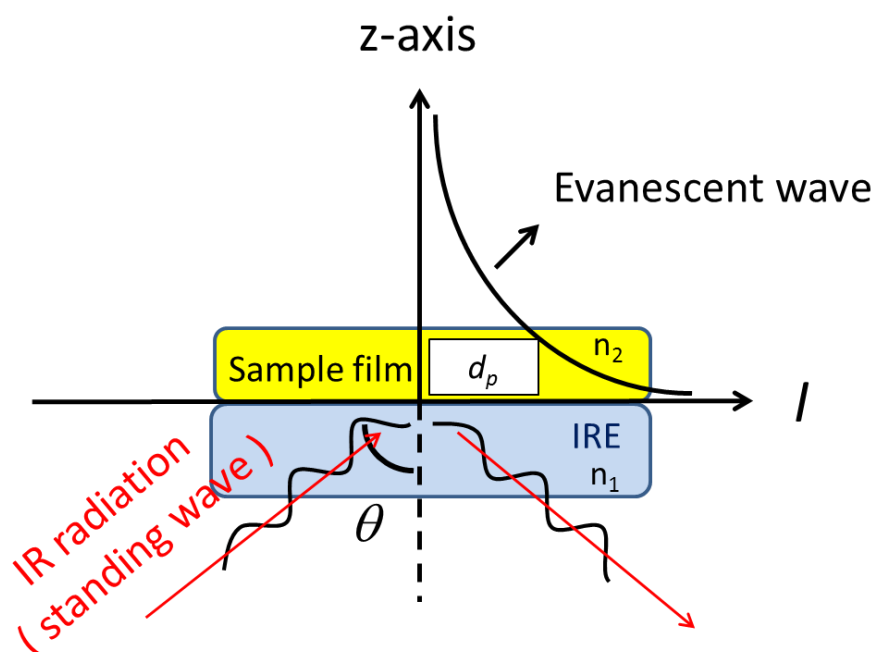


Figure 2-17. Schematic illustration of evanescent wave of IR radiation formed at the interface between an internal reflection element (IRE) and a sample film.

2.5.2 Spectroelectrochemical flow cell

Figure 2-18 depicts spectroelectrochemical apparatuses and the configuration used in PFIRE experiments. A silicon (Si) internal reflection element (IRE) is embedded into a polyether ether ketone (PEEK) baseplate, on which aliquots (1 μ L throughout) of carbon particle (with adsorbed enzyme) suspension are deposited to make a sample film with a piece of carbon paper sitting on the top. A home-built ATR-IR spectroelectrochemical flow cell (designed by Ricardo Hidalgo and Ian McPherson in the group) is assembled on the top and filled with buffer solution. The cell is made of PEEK, housing a reference electrode (RE) of miniature saturated calomel electrode (SCE, home-built), a platinum wire counter electrode (CE, Surepure Chemicals), and a graphite rod (0.9 mm diameter, Pentel) for connection to the crocodile clip and in contact with the carbon paper (Toray, TGP-H-030) pressed on the top of the particle film as a working electrode (WE). The cell is also equipped with two more ports, which are able to be connected to the flow system, consisting of a peristaltic pump (QL-1000, Williamson) and two pieces of tubing (1 mm diameter, kinetic technologies) for flow inlet and outlet. Efficient solution flow in our spectroelectrochemical cell means that we can introduce inhibitors or substrates during an experiment readily.

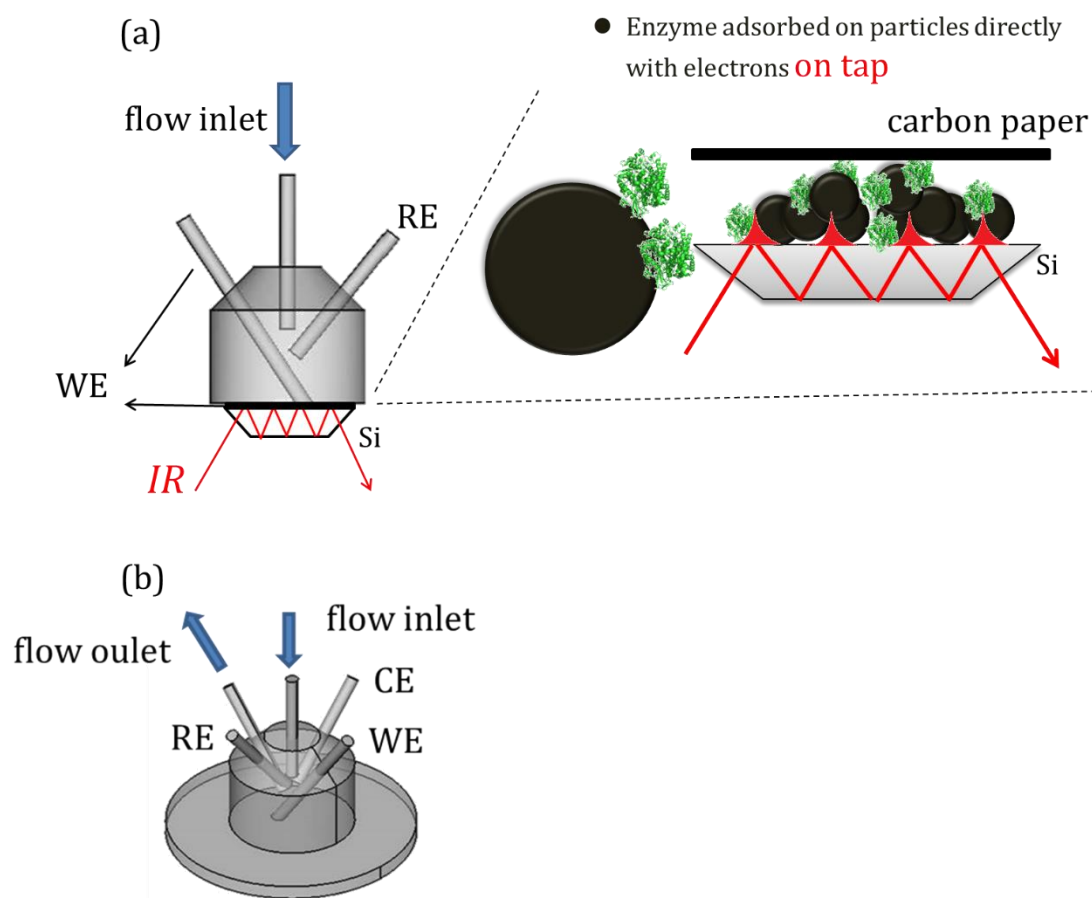


Figure 2-18. Diagrams of the PFIRE configuration. (a) Side view of the spectroelectrochemical flow cell showing a Si multi-bounce IRE and a carbon particle working electrode film (with adsorbed enzyme) deposited on the IRE in the expansion. (b) A 3D CAD representation of the spectroelectrochemical flow cell showing a baseplate, relative positions of the three electrodes, flow inlet and outlet.

2.5.3 Challenges in PFIRE

The prerequisite factor to make PFIRE work for studies of hydrogenase chemistry is that there must be enough enzyme immobilised on the carbon particles for FTIR sampling. In this Thesis, carbon nanoparticles with high surface area are used as the working electrode. The material selected for utilisation is commercial available carbon black, Black Pearls 2000 (BP2000), from Cabot Corporation. The specific surface area of it is $1487 \text{ m}^2 \text{ g}^{-1}$ BET.⁹⁹

It was found that high loading of *E. coli* Hyd-1 on BP2000 can be achieved easily by simply mixing enzyme solution with well dispersed particle suspension at low temperature (4 °C), which gives a good signal to noise ratio in the FTIR spectrum for analysis without the need for specific surface modification.⁵¹ However, *E. coli* Hyd-2 cannot be immobilised on BP2000 to a high loading for spectroscopic detection in this straightforward way; a pre-treatment of the carbon surface is required by surfactant to create a hydrophobic environment for immobilisation (see Chapter 3 for more details).

2.5.4 Particle preparations of *E. coli* Hyd-1 and Hyd-2 for PFIRE

A potassium phosphate buffer solution (15 mM, pH 5.8) was used for particle preparations throughout.

Hyd-1

A 15 μL aliquot of Hyd-1 solution (approximately 18 mg mL^{-1}) was mixed with 5 μL aliquots of BP2000 particle suspension (20 mg mL^{-1} ; well dispersed was achieved by sonication for 1 hour) and left at 4 °C for 3 days. The particles were then washed with buffer solution to give a final dispersion, also approximately 20 mg mL^{-1} . A 1 μL aliquot of this particle suspension was then deposited on the silicon IRE and allowed to dry partially before assembling the cell on the top.

Hyd-2

Step A: preparation of surfactant(DDAB)-modified BP2000

A 5 μL aliquot of BP2000 particle suspension (20 mg mL^{-1} ; well dispersed was achieved by sonication for 1 hour) was mixed with 5 μL aliquots of DDAB solution (10 mM), and the mixture was left at 4°C overnight. Removal of excess DDAB was carried out by centrifuging particles to take out the supernatant. The particles were then washed with 100 μL aliquots of buffer solution and collected by centrifugation (this process was repeated for three times) before further use for Hyd-2 adsorption.

Step B: Immobilisation of Hyd-2

A 200 μL aliquot of Hyd-2 solution (approximately 1.5 mg mL^{-1}) was mixed with the DDAB-modified BP2000, and left at 4 °C overnight for adsorption. Removal

of unadsorbed Hyd-2 was carried out by centrifuging particles to take out the supernatant. The particles were then washed to give a final dispersion, also approximately 20 mg mL⁻¹. A 1 μ L aliquot of this particle suspension was then deposited on the silicon IRE and allowed to dry partially before assembling the cell on the top.

2.5.5 PFIRE in practice

PFIRE experiments were carried out inside the modified anaerobic glove box (Glove Box Technology) using a Varian 680-IR spectrometer equipped with a GladiATR™ accessory (PIKE technology, custom modified). The glovebox was filled with a N₂ atmosphere and was maintained at a dew point of *ca.* 75-85 °C to minimise contributions of water vapour to the infrared spectrum. Data collections were performed using Varian Resolutions Pro 4.0 software. Spectra were recorded at 4 cm⁻¹ resolution and are reported as an average of 1000 scans. Electrochemical control was provided by an Autolab PGSTAT 128N potentiostat manipulated by Nova software (EcoChemie). Potentials are quoted with respect to SHE using the correction $E_{\text{SHE}} = E_{\text{SCE}} + 0.242 \text{ V}$ at 25 °C.⁸⁹ Gas flow controllers (Brooks Instrument) are equipped with the cell to provide accurate mixtures of gases.

An “absolute” spectrum presented in this Thesis is recorded and processed against a background spectrum of a clean Si IRE (recorded prior to the experiment). And a “difference spectrum” is calculated by subtracting one absolute spectrum from another, which is able to highlight the changes triggered by introduction of an inhibitor or application of a potential step. Spectral processing was carried out using Origin 9.1. A baseline was subtracted from the data using an interpolated spline function; care was taken to avoid distorting peak shapes through the choice of baseline anchors with reference to corresponding 2nd derivative spectra. Peaks in the ν_{CO} region of baseline-corrected spectra were fitted to Gaussian profiles (Figure 4-3 and Figure 4-4).

2.6 Experimental

2.6.1 Protein

Samples of wild-type Hyd-1 and Hyd-2 from *Escherichia coli* were purified according to a published protocol,⁶⁷ and were prepared by Elena Nomerotskaia. Both proteins were stored at -80 °C in a freezer, and thawed slowly before use.

The ¹³CO-labelled active site variant of Hyd-1 was purified by adapting a method used for producing isotope-labelled active site variant of Hyd-2.¹⁰⁰ The medium used for culturing bacteria is Werkman minimal medium containing nickel and iron salts, 0.4% glucose and 0.4% fumarate as carbon sources.¹⁰¹ Cells were grown in the presence of 0.4% ¹³CO gas in the culture headspace.

2.6.2 Gases, reagents and solutions

Gases

The gases used in experiments were of high purity:

H₂ (Premier Plus, Air Products); CO (BOC); N₂ (BOC); Ar (BOC).

Reagents

All reagents were of at least analytical grade.

Potassium cyanide (Fisher); Ethyl isocyanide (Hansa Fine Chemicals GmbH); *n*-butyl isocyanide (Sigma-Aldrich); *t*-butyl isocyanide (Sigma-Aldrich).

Methyl isocyanide was synthesised following a method reported by Schuster *et al*,¹⁰² and was prepared by Pathinan Paengnakorn.

Buffer solutions

A potassium phosphate buffer solution (15 mM, pH 5.8, BDH) was used for protein samples and particle preparations. For PFE and PFIRE experiments carried out at pH 7, a HEPES buffer solution was used, which refers to 4-[2-hydroxy-ethyl]-1-piperazine-N-2-ethanesulfonic acid (Fisher), containing 100 mM NaCl (Sigma) as supporting electrolyte. For experiments carried out at pH 6, a mixed buffer solution was prepared according to a published procedure, which contains 100 mM NaCl (Sigma-Aldrich), 15 mM MES (Fisher), 15 mM HEPES (Fisher), 15 mM TAPS (Fisher), 15 mM CHES (Fisher), and 15 mM sodium acetate (Fisher). Ultra high purity water (MilliQ Millipore, 18 M Ω cm) was used throughout.

Chapter 3: Development of Electrically Conducting Particles with High *E. coli* Hyd-2 Loading for Use in Protein Film Infrared Electrochemistry

In this chapter, three different strategies to immobilise *E. coli* Hyd-2 on carbon nanoparticles with high surface area are discussed. Surfactant-modified carbon nanoparticles are shown to be an effective way to achieve Hyd-2 immobilisation, overcoming the main challenge of low coverage of sample on an electrode for spectroscopic detection by ATR-IR spectroscopy. The focus of enzyme immobilisation on carbon material instead of on a metal surface avoids the possibility of the electrode reacting with solutes such as CN^- , which can be introduced into the cell during an experiment to study inhibition.

3.1 Introduction

3.1.1 Unmediated redox control of *E. coli* Hyd-2 coupled with ATR-IR spectroscopy

ATR-IR spectroscopy coupled with electrochemistry on Hyd-2 was carried out by means of a “particle network electrode” at a very beginning of this project. In the set-up of particle network film, an aqueous Nafion suspension (titrated to neutral pH) was exploited as polymer electrolyte and binder to trap enzyme molecules with carbon black particles for electrochemical control (enzymes are not immobilised on carbon particles for direct electrochemistry, Figure 3-1), which are then deposited on the Si IRE. This configuration has been demonstrated a facile method for studying redox metalloproteins without the need for a cocktail of solution mediators,¹⁰³ although an equilibration time is still required after each step in potential to allow proteins to diffuse to the electrode for electron exchange.

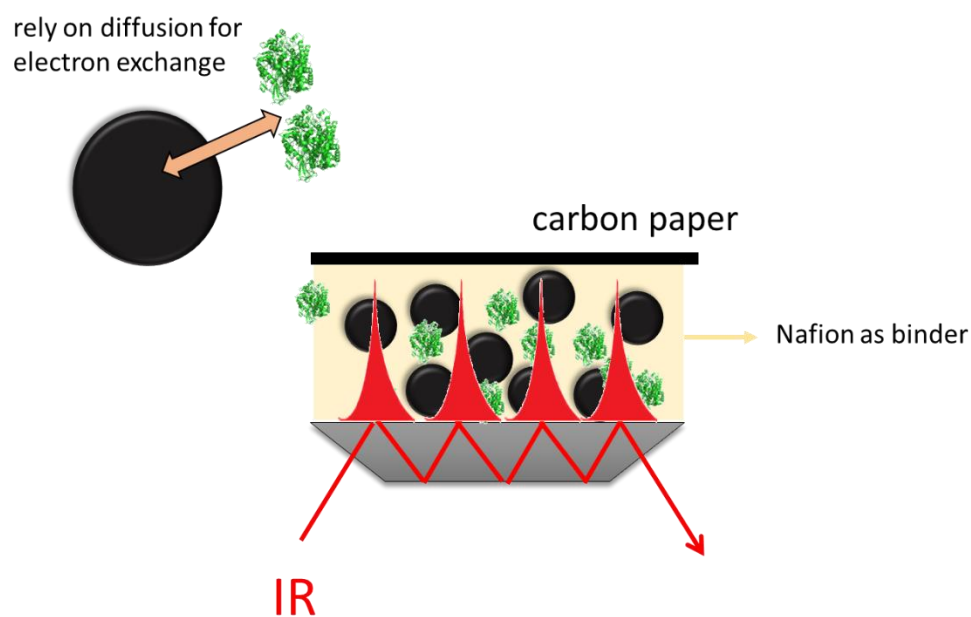


Figure 3-1. Schematic representation of a particle network electrode, constructed by using an aqueous Nafion suspension (titrated to neutral pH) as polymer electrolyte and binder to trap enzyme molecules with carbon black particles. The electrochemical control over enzyme relies on the diffusion process.

Chapter 3. Development of a particle electrode with high Hyd-2 loading

Figure 3-2 shows redox-triggered difference spectra of Hyd-2, recorded under N₂ as the potential was stepped to more positive values in 0.1 V intervals up to 0 V by the method mentioned above (background recorded at -0.4 V). The spectra presented were recorded 20 min after each potential step, at which point the enzyme was found to have reached diffusion-controlled equilibrium with the electrode potential. Only the changes in absorbance attributed to the endogenous CO ligand, ν_{CO} , are well resolved (2000-1900 cm⁻¹ region). Steps from -0.4 V to 0 V resulted in the appearance of a negative band at 1964 cm⁻¹, consistent with loss of the Ni-C state, and growth of a band at 1946 cm⁻¹, consistent with formation of Ni-SI_a (see Chapter 4 for redox state characterisations and detailed discussion).

Although conversions between states in response to application of potential steps can be achieved by this method, the formation of oxidatively inactive state, Ni-B, is not observed at high potentials, such as at 0 V. This is inconsistent with investigations from PFE (*cf.* Figure 2-4). Such a phenomenon might be attributed to the fairly slow process of Ni-B formation from oxidation of the Ni-SI_a state,⁴⁸ and it is much harder to be achieved when enzyme molecules have to rely on diffusion to reach equilibrium with the electrode potential. The inconsistent observation highlights the importance of studying redox enzymes by direct electrochemistry.

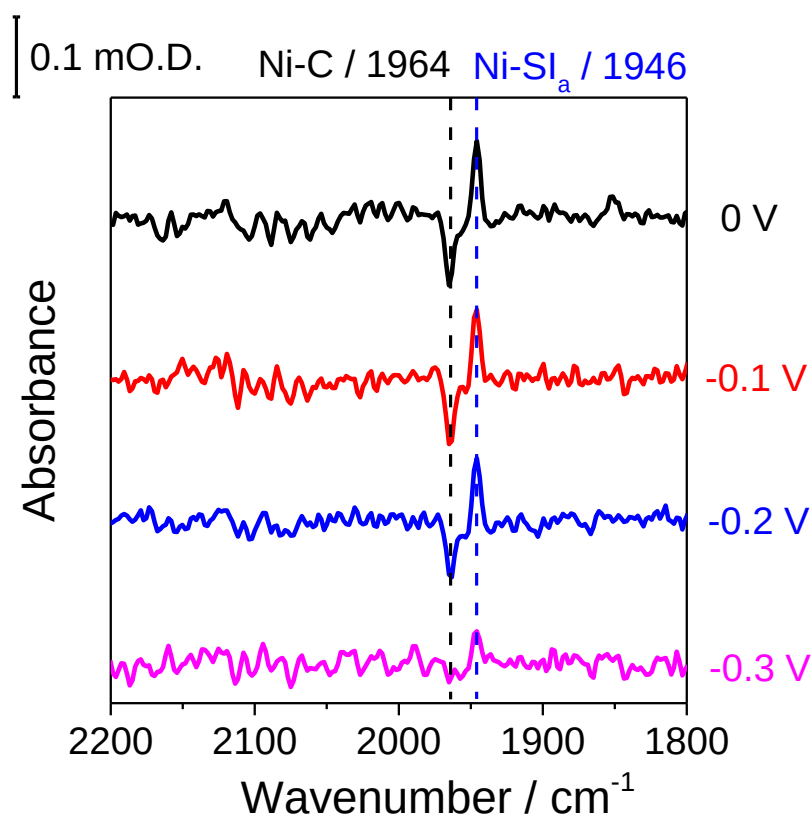


Figure 3-2. Redox-triggered difference spectra of Hyd-2 by means of a particle network electrode, in which pH-neutralised aqueous Nafion is used as binder. The background spectrum was collected at -0.4 V, followed by oxidative potential steps in 0.1 V intervals. Experimental conditions: 25 °C; pH 7; spectra were recorded in N₂-saturated buffer.

3.1.2 Protein film infrared electrochemistry

Protein film infrared electrochemistry (PFIRE) is a method coupling PFE with ATR-IR spectroscopy (see section 2.5 for more details), allowing direct electrochemical control of the enzyme and simultaneous collection of infrared spectra. The prerequisite aspect to have a good signal to noise ratio in FTIR spectra by this technique is to have a working electrode, which adsorbs the protein sample in large quantities.

E. coli Hyd-1 is found to be physically adsorbed on commercial carbon blacks with high surface area, BP2000 (Cabot Corp.), to a high loading by simply mixing enzyme solution with well dispersed particle suspensions at low temperature (4 °C). The use of the enzyme electrode, constructed from BP2000 (loaded with Hyd-1) deposited onto a Si IRE, has been demonstrated as a good approach to reveal hydrogenase chemistry.⁵¹ However, it is found that Hyd-2 cannot be adsorbed well enough to give sufficient loading on particles for spectroscopic detection in this straightforward way. Thus, much efforts were made to exploit different strategies to improve Hyd-2 loading on BP2000 for use in PFIRE, and the detail is presented in this Chapter.

3.1.3 Methods for examination of Hyd-2 adsorption

Among different immobilisation strategies, cyclic voltammetry on a RDE is exploited first to examine what condition enhances the adsorption of Hyd-2 on BP2000 most. The catalytic current is used as an indicator for comparison. Since each cycle of catalytic turnover for H₂ oxidation by a molecule of hydrogenase leads to electron flow into the electrode, the catalytic current is directly proportional to the number of electroactive enzyme molecules. Rapid rotation of the electrode during experiments provides efficient delivery of H₂ to the enzyme, avoiding substrate depletion in measurements. ATR-IR spectroelectrochemistry is then utilised to examine if the Hyd-2 loading is high enough for spectroscopic detection with satisfaction.

3.2 Physical adsorption of Hyd-2 on BP2000

3.2.1 Introduction

Interactions contributing to physical adsorption of an enzyme molecule on a support material mainly come from hydrophobic interactions and salt linkages (the combination of hydrogen bonding and electrostatic interactions).¹⁰⁴ In this section, the effect of pH is examined to see if a particular pH can enhance the amount of Hyd-2 immobilised on BP2000 via physical adsorption, as electrostatic interactions between particles and enzymes strongly depend on a given pH value of solution.

Hyd-2 has an isoelectric point (pI) of 4.5,¹⁰⁵ and generally commercial granular carbon materials have point of zero charge (PZC) of *ca.* 8.¹⁰⁶ Thus, three pH values, 4.5, 6, and 7, were chosen to prepare enzyme solutions and particle suspensions in this study. Dilute Hyd-2 solutions were used for particle preparations in RDE experiments (cyclic voltammetry), since minuscule amount of hydrogenase is enough to give a large catalytic current for analysis in electrochemistry. Once an optimised condition was found for Hyd-2 immobilisation on BP2000, more concentrated Hyd-2 solution was used to prepare particles for ATR-IR spectroelectrochemistry.

3.2.2 Experimental

Adsorption of Hyd-2 on BP2000

For cyclic voltammetry experiments on a rotating disk electrode (RDE), 5 μL aliquots of Hyd-2 solution (1.3 mg mL^{-1}) at pH 4.5, 6 and 7 were mixed with 5 μL well dispersed BP2000 suspension (20 mg mL^{-1}) at corresponding pH values, and left at 4 $^{\circ}\text{C}$ for 3 days. The particles were then washed with corresponding buffer solutions, and collected by centrifugation to give a final dispersion of approximately 20 mg mL^{-1} . For ATR-IR spectroelectrochemistry, the particle preparation was carried out in the same way as mentioned above, but was conducted with more concentrated Hyd-2 solution (20 μL aliquots of 8 mg mL^{-1} ; pH 6).

Cyclic voltammetry for examination of Hyd-2 adsorption

The catalytic currents of particles loaded with Hyd-2 were compared with that of enzyme adsorbed directly on a pyrolytic graphite edge (PGE) electrode. Although particles were prepared under different conditions, i.e. at different pH values, all electrochemistry experiments were conducted at pH 7 for comparison.

The electroactive film of Hyd-2 on a PGE electrode was prepared following the procedure described in section 2.2.3. As for particles loaded with Hyd-2, a 0.5 μL aliquot of the particle suspension was deposited on the PGE electrode,

and allowed to dry partially before immersing the electrode to enzyme-free buffer solution (pH 7) for experiments.

The adsorbed enzyme was activated under 1 bar H_2 by poisoning the potential at -0.56 V for 300 s between cyclic voltammetry scans (cycling between -0.56 and +0.342 V) until the maximum current reached in successive scans had stabilized, indicating that activation was complete. The working electrode was rotated rapidly (4000 rpm) to maintain the H_2 concentration at the electrode surface close to that in the bulk solution, so the catalytic current was not limited by mass transport. This was confirmed by the fact that no enhanced current occurred as the electrode rotation rate was increased.

3.2.3 Results and discussion

Cyclic voltammetry at a RDE electrode

Figure 3-3 shows cyclic voltammograms of immobilised Hyd-2 prepared under different conditions. A typical cyclic voltammogram of Hyd-2 adsorbed onto a bare PGE electrode is depicted in black, trace (a). Hyd-2 shows a positive/negative current crossover just above/below *ca.* -0.4 V (marked by a dashed vertical line in the inset of Figure 3-3), which corresponds well to the formal reduction potential of the H^+/H_2 couple (-0.413 V) under these conditions (1 bar H_2 ; pH 7; 25°C) predicted by the Nernst equation. This indicates that no significant overpotential is required for Hyd-2 catalysis when it is adsorbed on a bare PGE electrode.⁶⁷

Chapter 3. Development of a particle electrode with high Hyd-2 loading

Traces (b)-(d) depict catalytic currents of BP2000 particles loaded with Hyd-2, with the adsorption processes carried out at different pH values. Particles contributing to pink trace (b) were prepared at the pI of Hyd-2, pH 4.5. The catalytic currents (for both H₂ oxidation and production) are higher than that of directly adsorbing on a bare PGE electrode, i.e. the former has an oxidation current of *ca.* 17 μ A at +0.342 V, and the latter has a positive current of *ca.* 5 μ A at the same potential. The zero net current also happens at a potential of *ca.* -0.4 V, showing a negligible overpotential in H₂ oxidation/production is required when Hyd-2 is immobilised on BP2000 particles physically. Particles prepared with the adsorption process conducted at pH 6 give the highest current (*ca.* 35 μ A at +0.342 V) depicted in blue, trace (c). Adsorption carried out at pH 7 does not give that much Hyd-2 loading on BP2000 particles represented in red, trace (d), as the current at +0.342 V is just approximately twice as high as that of directly adsorbing on a PGE electrode, in spite of the much higher surface area of BP2000 particles.

When an adsorption process is conducted in the neighbourhood of the isoelectric point of the protein, it is expected that hydrophobic interaction will dominate the process. It has been reported that this condition gives good enzyme adsorption on a range of materials.¹⁰⁷ In the case of Hyd-2 on BP2000, immobilisation carried out at pH 4.5 (pI of Hyd-2) provides a moderate adsorption level, giving *ca.* 4 times current enhancement at +0.342 V compared with that of Hyd-2 adsorbing on a bare PGE electrode. The highest loading of enzyme on particles is achieved at pH 6, where the pH is lower than the PZC

(ca. pH 8) of granular carbon materials and higher than the pI of Hyd-2 (pH 4.5). Thus, it appears that the adsorption is favoured by electrostatic interaction between positive charges on carbon and negative charges on the protein. As pH goes higher to 7 for adsorption, the condition approaches the PZC of the carbon, and thus the driving force for enzyme immobilisation decreases.

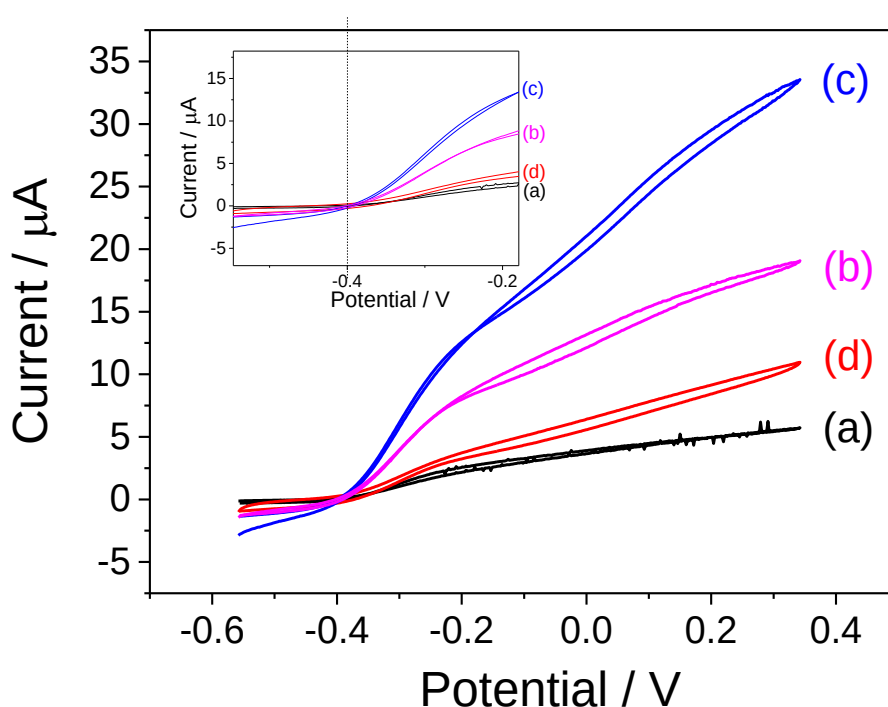


Figure 3-3. Cyclic voltammograms of Hyd-2 under 1 bar H₂. Trace (a): Hyd-2 is directly adsorbed on the bare PGE electrode. Trace (b)-(d): particles deposited on the PGE electrode with the adsorption process carried out at pH 4.5 (pink trace), pH 6 (blue trace) and pH 7 (red trace), respectively. Other conditions: scan rate, 10 mV s⁻¹; electrode rotation rate, 4000 rpm; 28 °C. The inset is an expansion of the region of the voltammograms, showing the potential of zero net current (marked by a dashed vertical line).

Chapter 3. Development of a particle electrode with high Hyd-2 loading
ATR-IR spectroelectrochemistry

On the basis of all electrochemical results described above, pH 6 was chosen for preparation of particles with more concentrated Hyd-2 solution for use in ATR-IR experiments.

Figure 3-4 depicts a cyclic voltammogram of Hyd-2 immobilised on BP2000 recorded in the spectroelectrochemical flow cell (ATR-IR cell). The potential was swept between -0.558 V and +0.342 V at a scan rate of 10 mV s⁻¹. H₂ saturated buffer was flowed rapidly into the particle film (46.2 mL min⁻¹), so the current was not limited by availability of H₂ to the enzyme. This was confirmed by the fact that no enhanced current was observed as the flow rate of H₂ saturated buffer was increased. The potential of zero net current also happens at *ca.* -0.4 V, which is consistent with the results of RDE experiments, namely there is no detectable overpotential in catalysis when Hyd-2 adsorbs on BP2000. However, the oxidation current at +0.342 V is only approximately 38 μ A (*cf.* Figure 3-3, blue trace (c)), indicating that no significant enhancement of Hyd-2 immobilisation on BP2000 occurred, even though the adsorption process was carried out with more concentrated enzyme solution.

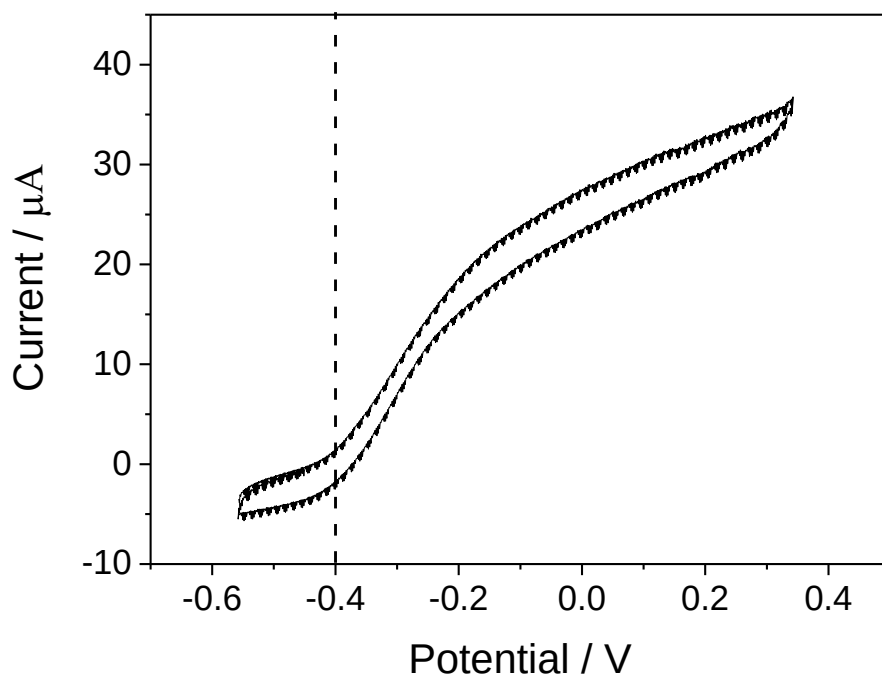


Figure 3-4. Cyclic voltammogram of Hyd-2 recorded in the ATR-IR cell. Other conditions: H_2 saturated buffer flow rate to the cell, 46.2 mL min^{-1} ; scan rate, 10 mV s^{-1} ; 25°C . The potential of zero net current is marked by a dashed vertical line.

Figure 3-5, panel (a) shows an absolute spectrum of as-isolated Hyd-2 immobilised on BP2000 under N₂. Clear absorptions at 1652 cm⁻¹ and 1545 cm⁻¹ arise from vibrations of amide I (mainly governed by C=O stretching) and amide II (mainly dominated by N-H bending) of groups in the protein. These two absorption bands provide evidence that Hyd-2 is immobilised on the carbon particles, although the amide I band is heavily convoluted with the water bending vibration at 1645 cm⁻¹. A broad and intense absorption band at *ca.* 3600-3000 cm⁻¹ is contributed by the OH stretching of water. Panel (b) shows an expanded view of 2100-1850 cm⁻¹ region of the spectrum shown in panel (a), where it is clear of solvent and protein vibrational bands. Thus, all absorption bands here arise from endogenous CO and CN⁻ ligands of the hydrogenase active site. A weak peak at 1957 cm⁻¹ is present in panel (b), which is attributed to ν_{CO} of a mixture of Ni-A and Ni-B states (see Chapter 4 for more details in peak assignments). Absorption bands of ν_{CN} of the active site (supposed to appear around 2080 cm⁻¹) are far less intense than ν_{CO} , and cannot be seen in the absolute spectrum as the amount of Hyd-2 adsorbed on BP2000 is not high enough. Panel (c) shows a difference spectrum of Hyd-2, which was recorded 4 hours after the potential being poised at -0.558 V under H₂ for activation, and is subtracted by the spectrum of panel (a). The appearance of a negative peak of ν_{CO} at 1957 cm⁻¹ and a positive peak of ν_{CO} at 1946 cm⁻¹ indicates that the electronic environment of Hyd-2 active site changes in response to the activation process; changes in the region of ν_{CN} are also visible.

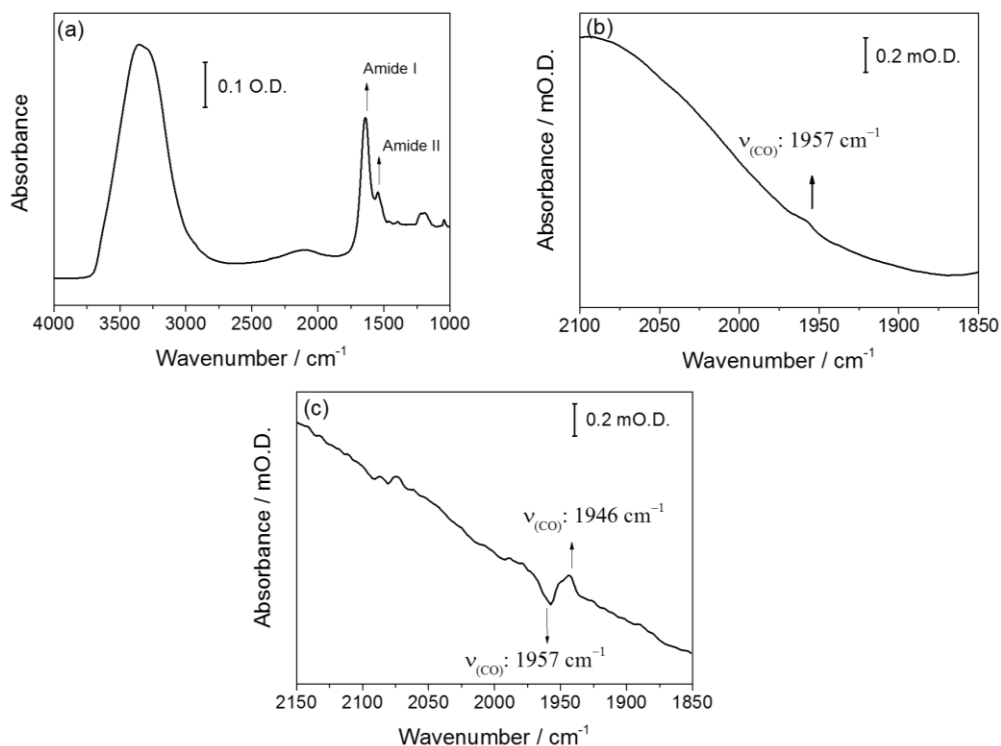


Figure 3-5. Panel (a): an absolute ATR-IR spectrum of as-isolated Hyd-2 loaded on the BP2000 particles under N_2 (the adsorption process was carried out at pH 6). Panel (b): an expanded view of spectrum (a), showing the vibrational region of endogenous CO and CN^- ligands of the active site. Panel (c): a redox-triggered difference spectrum of Hyd-2, which was recorded 4 hours after the potential being poised at -0.558 V under H_2 for activation, and was subtracted by the spectrum of panel (a).

3.2.4 Summary

In this section, changes of pH are proved to have an influence on the amount of Hyd-2 immobilised on BP2000 particles via physical adsorption. Maximum adsorption is achieved at pH 6, and the process appears to be governed by electrostatic interactions between the enzymes and the carbon particles. However, the use of more concentrated Hyd-2 solution under the condition of pH 6 for immobilisation does not give satisfactory results in ATR-IR experiments. The oxidation current depicted in a cyclic voltammogram is low and the S/N is not good enough in the FTIR spectra for detailed studies. Thus an alternative method is needed to prepare particles loaded with Hyd-2.

3.3 Covalent bonding of *E. coli* Hyd-2 onto carbon nanoparticles

3.3.1 Introduction

In this section, a method of immobilising Hyd-2 on carbon particles via covalent bonding is used to examine if it can give good results in ATR-IR spectroelectrochemistry. Figure 3-6 depicts the strategy. It starts with introduction of amine groups on the carbon particle surface (abbreviated as BP2000-NH₂ throughout this section). Then a coupling reagent, glutaraldehyde (a homobifunctional crosslinker), serves as a spacer arm, linking the enzymes to the carbon surface via reactions with the amine groups.

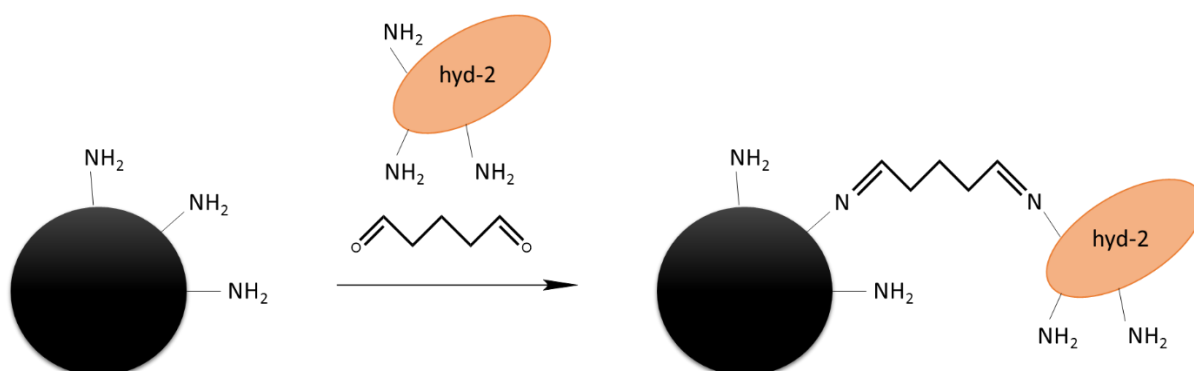


Figure 3-6. Schematic representation of the immobilisation strategy via covalent bonding used in this section.

3.3.2 Results and discussion

Introduction of amine groups onto the BP2000 carbon surface

Surface modification of carbon particles with amine groups was achieved by the reduction of nitrate functionalised carbon black, which is adapted from a method reported by Tsubokawa *et al.*¹⁰⁸ To produce BP2000 functionalised with nitrate groups on the surface, a suspension of 100 mg of BP2000 in 5 mL of a mixture of concentrated sulphuric and nitric acids (3:1 ratio by volume, 98% and 70%, respectively) was prepared at room temperature. The suspension was subjected to sonication for 9 hours, and carbon particles were then centrifuged and washed with distilled water until the supernatant was at neutral pH. To achieve modification of the carbon particle surface with amine groups, the nitrate functionalised carbons were then added to a 25 mL solution containing 2.2 mL of 35% ammonia water and 8.8 mL of distilled water, and 14 mL of 500 mM sodium dithionite solution. The reaction mixture was stirred gently at room temperature for 24 hours. The carbon black was then centrifuged and washed with distilled water until the supernatant was at neutral pH. The resulting carbon black was dried in an oven.

For inspection of modification after each step, a 1 μL aliquot of particle suspension (74 mg mL^{-1}) was deposited on the Si IRE and allowed to dry completely for direct measurements. Figure 3-7 depicts ATR-IR spectra collected after each modification step with a spectrum of the clean prism as reference (recorded prior to the measurements). Trace (a) shows a spectrum of

BP2000 used as received, and reveals that there is no strong characteristic infrared absorption bands. A spectrum collected after BP2000 being subject to sonication in a mixture of concentrated sulphuric acid and nitric acid is presented in red, trace (b). Two intense absorption bands at 1576 and 1432 cm^{-1} appear, contributed by vibrations of nitro groups on the surface.¹⁰⁹ Reduction of the nitrate functionalised particles gives a spectrum shown in blue, trace (c). The peak at 1576 cm^{-1} of the nitrate functionalised particles shifts to 1563 cm^{-1} , indicating a success in introduction of amine groups.¹⁰⁹

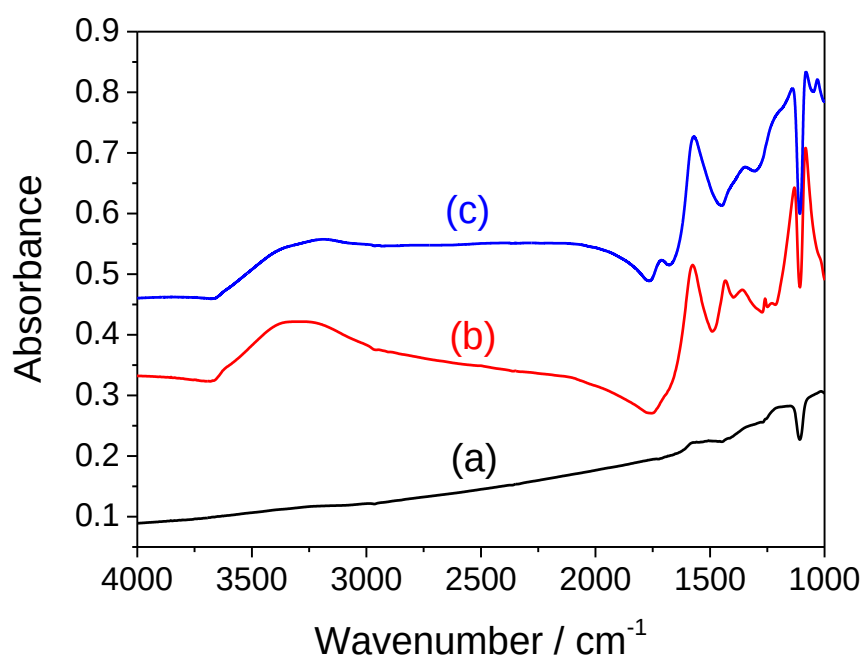


Figure 3-7. ATR-IR spectra of dry particle films; a spectrum of the clean prism is used as reference: trace (a), carbon black BP2000 (used as received); trace (b), nitrate functionalised BP2000; and trace (c), BP2000 modified with amine groups on the surface (BP2000- NH_2).

Immobilisation of Hyd-2 via covalent bonding

A potassium phosphate buffer solution (15 mM, pH 7.4) was used throughout. Immobilisation of Hyd-2 on BP2000 via covalent bonding was carried out as follows: a 30 μL aliquot of BP2000-NH₂ from 74 mg mL⁻¹ dispersion was mixed with glutaraldehyde (2.5 %) in a total volume of 100 μL . The suspension was stirred gently for 11 hours at room temperature for activation. After the activation process, particles were washed with buffer solution to give a final dispersion of *ca.* 74 mg mL⁻¹. The activated BP2000-NH₂ particles must be used immediately after preparation. A 10 μL aliquot of activated BP2000-NH₂ particles (74 mg mL⁻¹) was mixed with 20 μL aliquots of Hyd-2 solution (10 mg mL⁻¹) at room temperature for 1.5 hours. Particles loaded with Hyd-2 were then washed with buffer solutions to give a final dispersion of *ca.* 74 mg mL⁻¹ and stored at 4 °C for further experiments.

Evidence for Hyd-2 (as-isolated) attachment on the carbon surface via a coupling reagent, glutaraldehyde, is provided by ATR-IR spectroscopy (a 1 μL aliquot of particle suspension, 74 mg mL⁻¹, was deposited on the Si IRE), Figure 3-8. Two characteristic infrared absorption bands of a protein, 1652 cm⁻¹ (amide I) and 1545 cm⁻¹ (amide II) are clearly seen in panel (a), albeit the amide I band is overlapping with the water bending vibration at 1645 cm⁻¹ to some degree. Panel (b) expands the region (2150-1850 cm⁻¹) of spectrum (a), where endogenous CO and CN⁻ ligands of the hydrogenase active site are expected to be present. A weak peak at 1957 cm⁻¹ (ν_{CO}) is seen in panel (b), which is

contributed by a mixture of Ni-A and Ni-B states. The ν_{CN} bands of the hydrogenase active site are not observed, as the Hyd-2 loading is not high enough to resolve these peaks, which are far less intense compared with ν_{CO} .

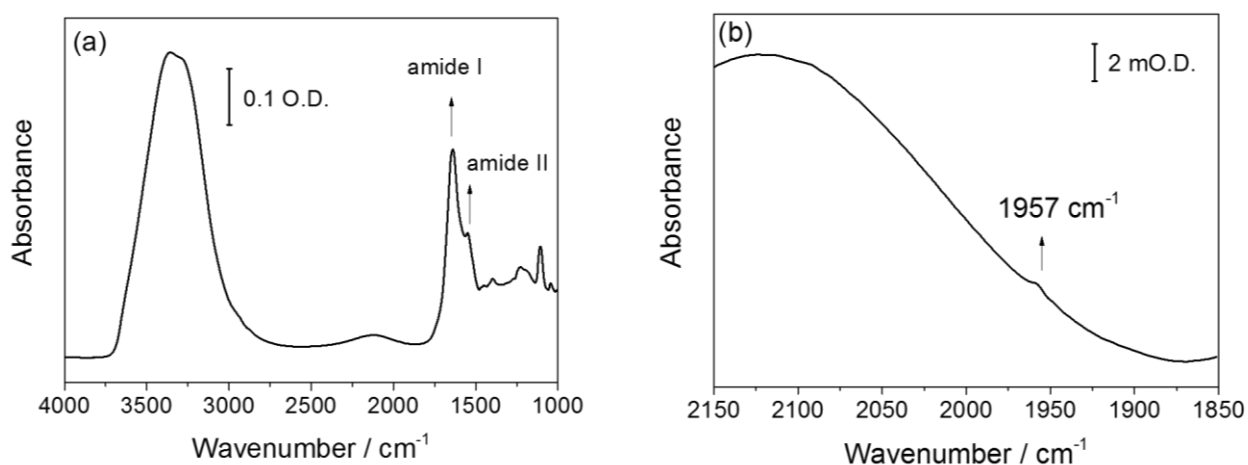


Figure 3-8. (a) An absolute ATR-IR spectrum of particles loaded with Hyd-2 (as-isolated) via covalent bonding. (b) An expanded view of spectrum (a), showing the vibrational region of intrinsic CO and CN^- ligands of the active site. Collection of the spectrum was carried out in N_2 -saturated buffer (pH 7).

Electrochemistry on a RDE

Cyclic voltammetry on a RDE was exploited for careful examination of the electrochemical properties of Hyd-2 immobilised on the carbon surface via covalent bonding, Figure 3-9. Black trace (a) shows a voltammogram of Hyd-2 adsorbed on a bare PGE electrode. The currents of both H₂ oxidation and proton reduction are not very high, i.e. oxidation current is just around 3.8 μ A at +0.342 V. The currents improve slightly in the measurement of depositing BP2000 loaded with Hyd-2 (the physical adsorption of Hyd-2 on BP2000 was carried out at pH 7) depicted in blue, trace (b). The oxidation current is *ca.* 11 μ A at +0.342 V. The onset potential for H₂ oxidation in (a) and (b) is at approximately -0.4 V, indicating that negligible overpotential is required for Hyd-2 in catalysis when it adsorbs on a bare PGE electrode and BP2000 particles (*vide supra*). A cyclic voltammogram of Hyd-2 immobilised on BP2000 carbon surface via covalent bonding is represented in red, trace (c). Although much higher concentration of Hyd-2 (10 mg mL⁻¹, 20 μ L) was used to prepare particles compared with that (1.3 mg mL⁻¹) for results shown in trace (a) and (b), the oxidation current is fairly low, i.e. just around 6 μ A at +0.342 V. In addition to the low catalytic current, when Hyd-2 links to the carbon surface via covalent bonding, an overpotential of approximately 70 mV is required for H₂ oxidation.

An explanation for such a low catalytic current and a requirement for overpotential in catalysis when Hyd-2 is immobilised on carbon particles via

covalent bonding is depicted in Figure 3-10 by a cartoon representation. Glutaraldehyde is a homobifunctional crosslinker, which reacts with amine groups on the carbon and the protein surface randomly to form a chemical bond. Therefore, not all Hyd-2 immobilised on the carbon surface will be in the right orientation for effective electron transfer between the carbon and the protein active site through the FeS clusters. In contrast, adsorptions of Hyd-2 on a PGE electrode and BP2000 physically are mainly governed by hydrophobic interactions (Albeit the electrostatic interaction can enhance the amount of adsorbed Hyd-2 slightly, section 3.2.), so the attachment point may be similar to that of linking Hyd-2 to a membrane under physiological conditions. This would lead to a correct orientation of Hyd-2 on the carbon surface without causing a significant overpotential in catalysis.

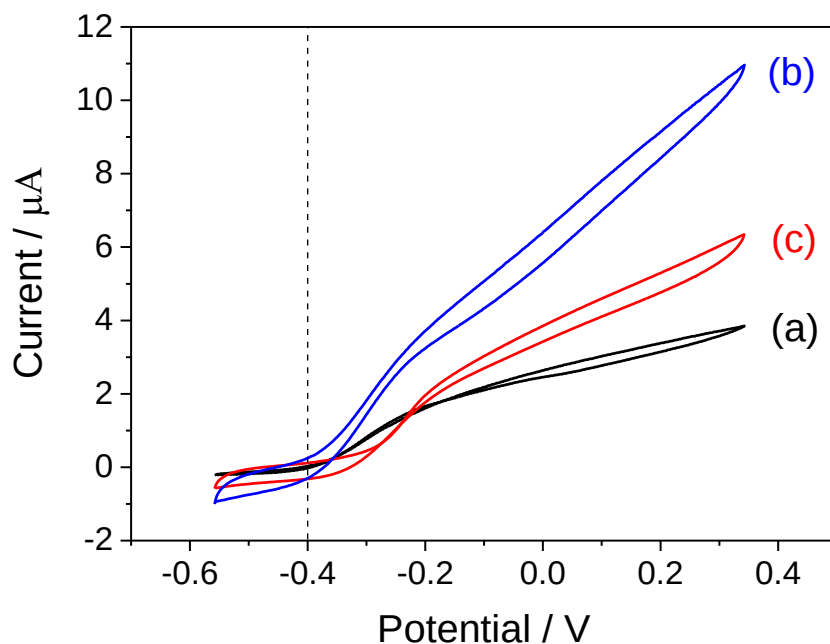


Figure 3-9. Cyclic voltammograms of Hyd-2 under 1 bar H_2 . Black trace (a): Hyd-2 is directly adsorbed on a bare PGE electrode. Blue trace (b): immobilisation of Hyd-2 on BP2000 via physical adsorption; the adsorption process was carried out at pH 7. Red trace (c): immobilisation of Hyd-2 on BP2000 via covalent bonding. Other conditions: pH 7; scan rate, 10 mV s^{-1} ; electrode rotation rate, 4000 rpm; 28°C .

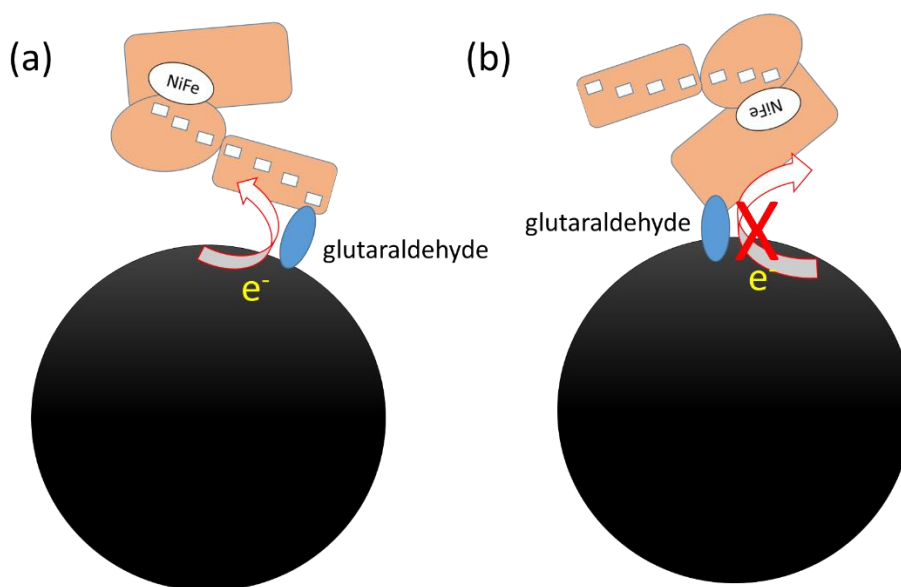


Figure 3-10. Cartoon representations of (a) effective electron transfer between the carbon and the Hyd-2 active site through the FeS clusters, and (b) immobilisation of Hyd-2 on the carbon surface in a wrong orientation. The coupling reagent of glutaraldehyde is shown as a blue ellipse, and FeS clusters are symbolised by squares.

3.3.3 Summary

In this section, structural evidence provided by ATR-IR spectroscopy shows that immobilisation of Hyd-2 on BP2000 via glutaraldehyde for covalent bonding is successful. However, further examination by electrochemistry on a RDE suggests that not all protein molecules immobilised on the carbon surface are in the right orientation for effective electron exchange between the carbon and the active site via FeS clusters, resulting in a low catalytic current and a requirement of significant overpotential in catalysis. Thus, covalent bonding is not an ideal strategy for use in protein film infrared electrochemistry if we want to study more detailed hydrogenase chemistry via this technique.

3.4 Reconstruction of a physiologically-mimicking condition on the carbon support for Hyd-2 immobilisation

3.4.1 Introduction

This section describes a strategy of using a surfactant, didecyltrimethylammonium bromide (DDAB), to build up a micelle on the carbon surface, which mimics a native lipid bilayer for Hyd-2 immobilisation via hydrophobic interaction. The idea comes from the fact that as-isolated Hyd-2 contains three subunits: HybC (large subunit harbouring the active site), HybO (small subunit accommodating three FeS clusters) and HybA (containing four ferredoxin-like clusters), predicted by sequence analysis.¹¹ HybO and HybA have a large footprint with hydrophobic feature. Figure 3-11 (a) and (b) depict how Hyd-2 associates with a membrane under physiological conditions, and a SDS-PAGE gel of purified Hyd-2, showing that the as-isolated protein contains three subunits.

The cationic surfactant DDAB is chosen in development of this method, as it can form a biomembrane-mimic structure on solid supports spontaneously, which is water dispersible.¹¹⁰ This property of DDAB has been widely used in several biomedical applications, such as DNA transfection,¹¹¹ and in direct electrochemical studies of haemoglobin¹¹² and cytochrome monooxygenase P450.¹¹³ Figure 3-11 (c) shows a cartoon representation of how a physiologically-mimicking environment is reconstructed on BP2000 for Hyd-2

adsorption in the correct orientation for effective electron transfer developed in this section.

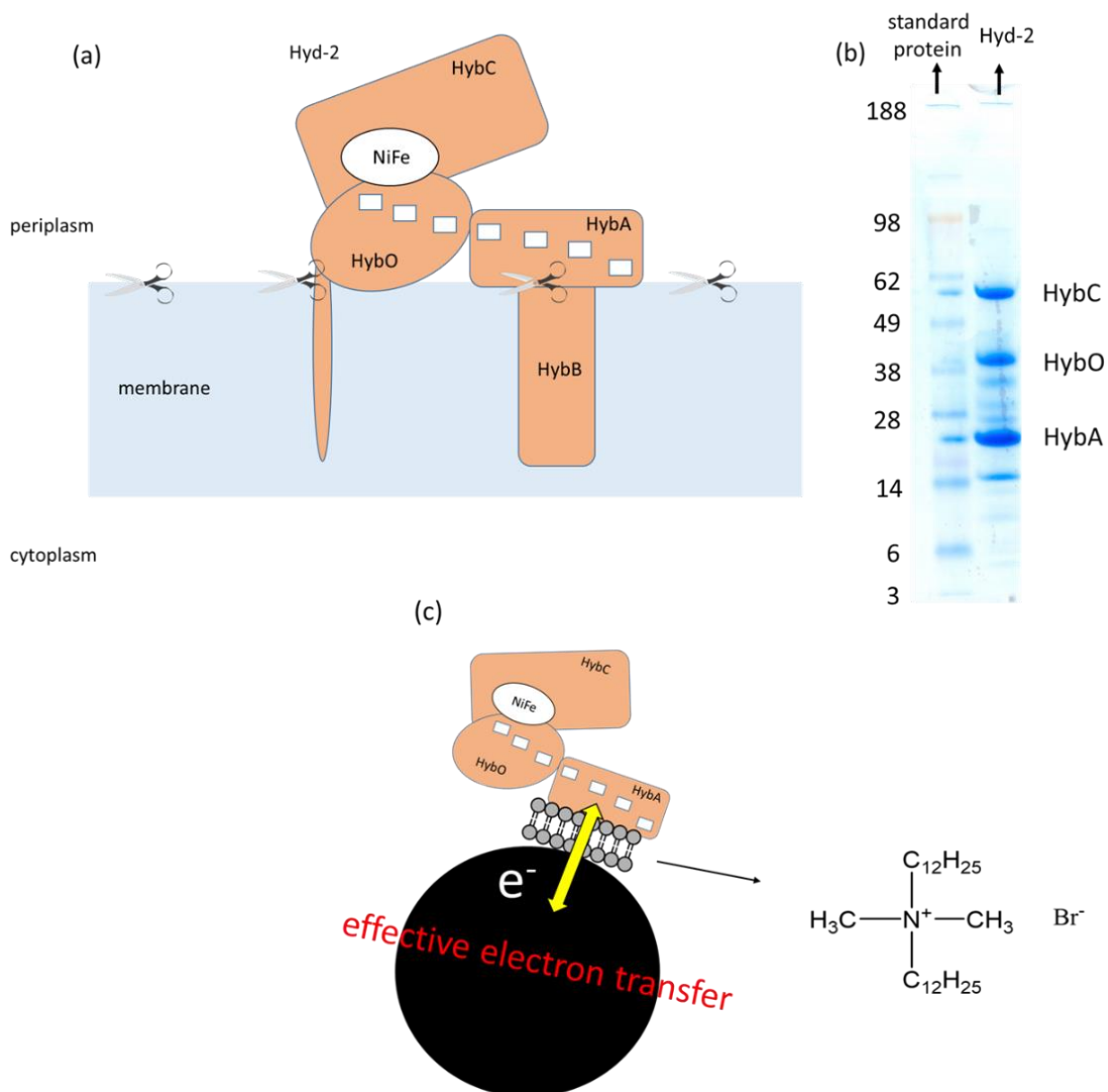


Figure 3-11. (a) A cartoon representation of native Hyd-2 in *E. coli* associating with a membrane; the structure is predicted by sequence analysis.¹¹ The detachment point is denoted by scissors, when a detergent is exploited to release the membrane-associated Hyd-2 during purification. FeS clusters are represented by squares. (b) A SDS-PAGE gel of purified Hyd-2, showing the as-isolated protein contains three subunits. (c) A cartoon representation of the

concept used in this section: reconstruction of a physiologically-mimicking environment for Hyd-2 adsorption by the surfactant, DDAB, which forms stable amphiphilic membranes on the carbon surface. Thus, adsorption of Hyd-2 on carbon is facilitated by hydrophobic interactions.

3.4.2 Results and discussion

Preparation of surfactant-modified BP2000

The process of forming a lipid bilayer structure on a support material is governed by van der Waals and electrostatic interactions.¹¹⁴ In this section, a potassium phosphate buffer solution with low ionic strength (15 mM, pH 5.8) was used throughout to start with. The same buffer solution was also used for storage of the as-isolated Hyd-2 after the purification.

A 5 μL aliquot of well dispersed BP2000 particle suspension (20 mg mL^{-1}) was mixed with 5 μL aliquots of DDAB solution (10 mM) and the mixture was left at 4°C overnight. Removal of excess DDAB was carried out by centrifuging particles to take out the supernatant. The particles were then washed with 100 μL aliquots of buffer solution, and collected by centrifugation (repeated for three times) to give a final dispersion of 20 mg mL^{-1} before further use for Hyd-2 adsorption. Figure 3-12 shows the spectrum of dry BP2000 particles modified with DDAB surfactant. The asymmetric and symmetric $-\text{CH}_2$ stretches at 2923 and 2850 cm^{-1} of DDAB are clearly seen,¹¹⁵ providing evidence of the successful modification. A broad absorption band at *ca* 3600-3000 cm^{-1} is contributed by the OH stretch of water trapped in the micelles.

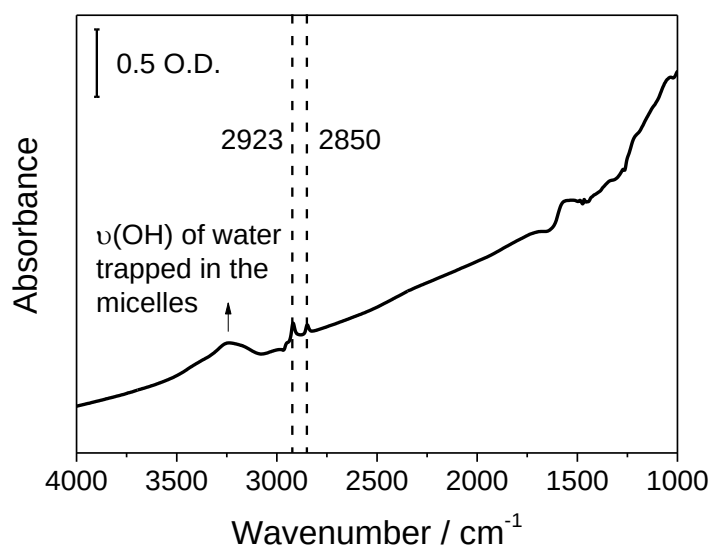


Figure 3-12. An absolute ATR-IR spectrum of dry BP2000 particles modified with DDAB surfactant. A spectrum of the clean prism was used as reference.

Adsorption of Hyd-2 on the surfactant-modified BP2000

A 200 μL aliquot of Hyd-2 solution (1.5 mg mL^{-1}) was mixed with 5 μL aliquots of DDAB-modified BP2000 suspension (20 mg mL^{-1}), and left at 4°C overnight for adsorption. Removal of unadsorbed Hyd-2 was carried out by centrifuging particles to take out the supernatant. The particles were then washed with 50 μL aliquots of buffer solution and collected by centrifugation (repeated for three times) to give a final dispersion of approximately 20 mg mL^{-1} . A 1 μL aliquot of this suspension was deposited on the Si IRE and allowed to dry partially; the spectroelectrochemical flow cell was then assembled on the top, filled with N_2 -saturated buffer (pH 7) for ATR-IR spectroelectrochemistry.

Two intense characteristic bands of a protein, 1652 cm^{-1} (amide I) and 1545 cm^{-1} (amide II) provide evidence of particles being loaded with Hyd-2 (as-isolated) successfully (Figure 3-13, panel a). Peaks contributed by the DDAB surfactant are also clearly seen (the asymmetric and symmetric $-\text{CH}_2$ stretching bands at 2923 and 2850 cm^{-1}), marked by a red circle (*cf.* Figure 3-12). Panel (b) expands a region of the intrinsic CO and CN $^-$ ligand stretches of spectrum (a), where the active site peaks are clearly seen with a good S/N. Processing spectrum (a) with baseline correction gives spectrum (c), in which an intense absorption band at 1957 cm^{-1} (ν_{CO}) contributed by a mixture of Ni-A and Ni-B states is 0.6 mO.D in absorbance (see Chapter 4 for more details in peak assignments). Peaks attributed to ν_{CN} are also clearly visible, with the

asymmetric and symmetric vibrational bands of the two endogenous cyanide ligands at 2092 and 2081 cm^{-1} , respectively.

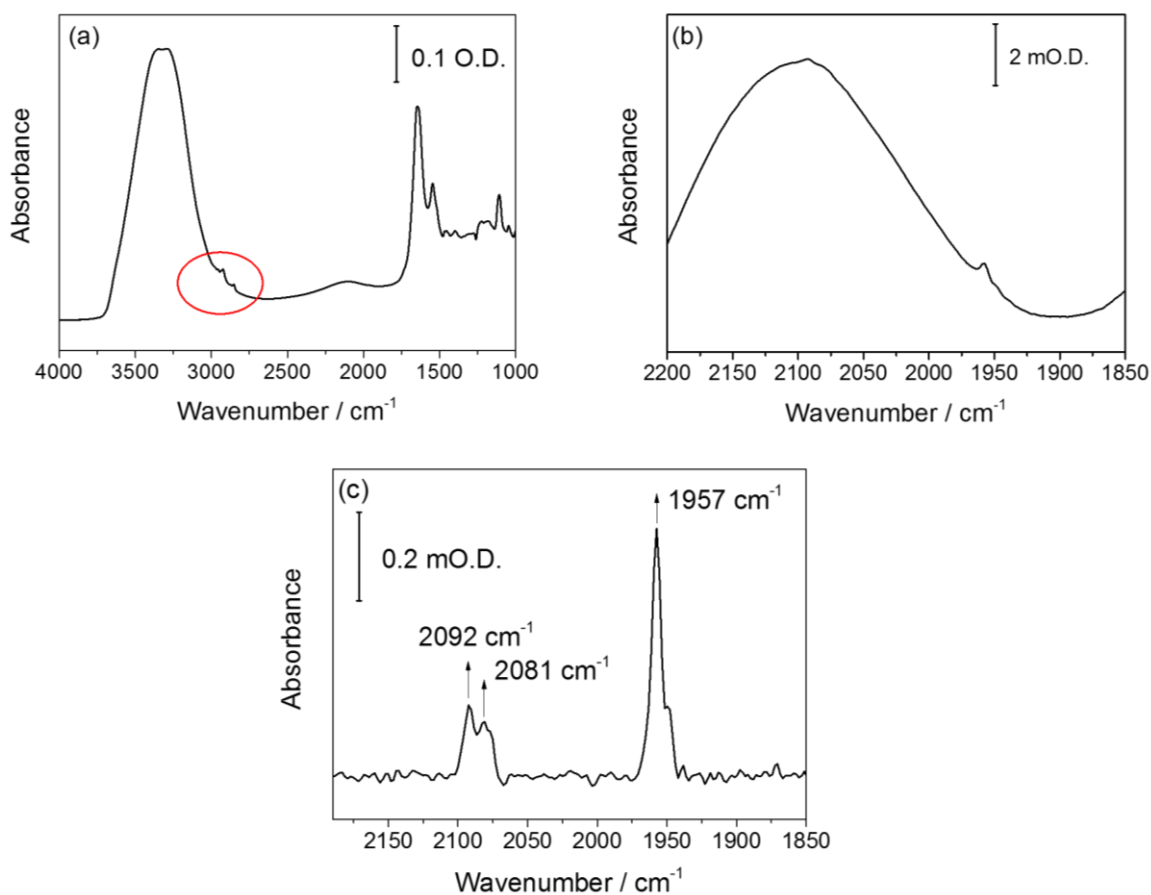


Figure 3-13. Panel (a): an absolute ATR-IR spectrum of DDAB-modified particles loaded with Hyd-2 (as-isolated). Peaks marked by a red circle are absorption bands of $-\text{CH}_2$ in DDAB. Panel (b): an expanded view of spectrum (a), showing the stretching region of intrinsic CO and CN^- ligands. Panel (c): a spectrum obtained from baseline subtraction of spectrum (a).

Chapter 3. Development of a particle electrode with high Hyd-2 loading
ATR-IR spectroelectrochemistry

Figure 3-14, panel (a) shows cyclic voltammograms collected in the ATR-IR cell. High catalytic currents are obtained for both H₂ oxidation (in H₂-saturated buffer, pH 7) and proton reduction (in Ar-saturated buffer, pH 7), i.e. the oxidation current of *ca.* 300 μ A at +0.2 V (H₂-saturated buffer solution flow rate, 46.2 mL min⁻¹), and the reduction current of *ca.* -75 μ A at -0.6 V (Ar-saturated buffer solution flow rate, 46.2 mL min⁻¹). The fact that the currents are not limited by mass transport was confirmed by observations that no enhancement in current occurred as the flow rate was increased. The zero net current of H₂ oxidation happens at a potential of *ca.* -0.4 V (marked by a dashed vertical line), which also corresponds well to the formal reduction potential of the H⁺/H₂ couple (-0.413 V) under these conditions (1 bar H₂, pH 7, 25°C) predicted by the Nernst equation. This indicates that no significant overpotential is required for Hyd-2 in catalysis when it adsorbs on DDAB-modified particles. Panel (b) depicts a difference spectrum of Hyd-2, which was recorded 3 hours after the potential being poised at -0.558 V under H₂ for activation, and is subtracted by the spectrum of as-isolated Hyd-2 (recorded under Ar). The appearance of a negative peak of ν_{CO} at 1957 cm⁻¹ and positive peaks of ν_{CO} at 1964 and 1949 cm⁻¹ indicates that the electronic environment of Hyd-2 active site changes in response to the activation process. Changes in the region of ν_{CN} are also clearly visible. Detailed assignments of redox states and vibrational bands of endogenous CO and CN⁻ ligands are described in more detail in Chapter 4.

Chapter 3. Development of a particle electrode with high Hyd-2 loading

A good S/N in ATR-IR spectra and large catalytic currents (Figure 3-14) report a high loading of Hyd-2 on the surfactant-modified BP2000, which is presumably facilitated by hydrophobic interactions. A biomembrane-mimic structure on the carbon surface leads to adsorption of Hyd-2 in the correct orientation for effective electron transfer, revealed by the observations of negligible overpotential requirement for H₂ cycling in voltammograms.

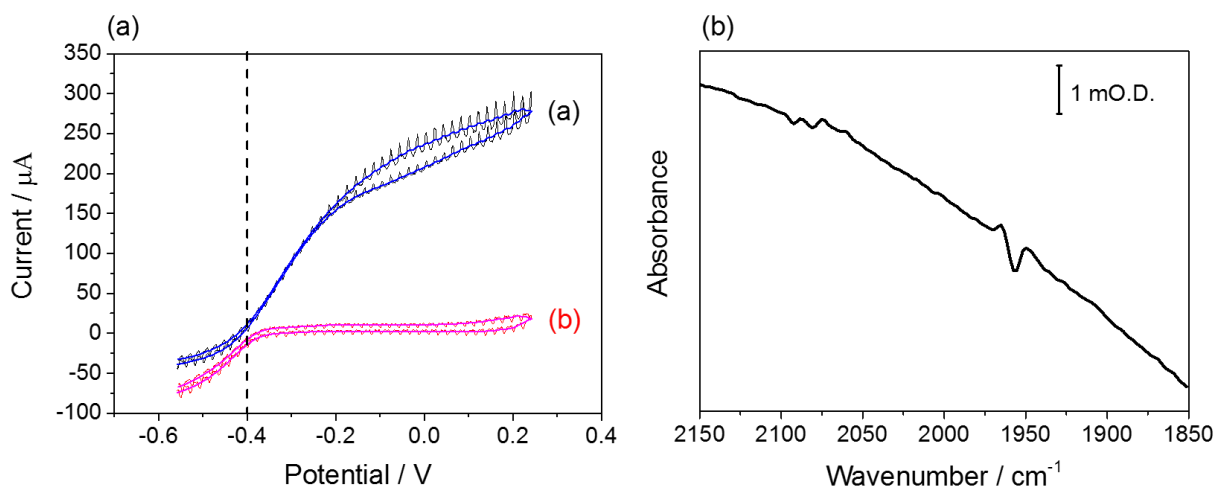


Figure 3-14. Panel (a): cyclic voltammograms collected in the ATR-IR cell; trace (a), recorded under 1 bar H_2 ; trace (b), recorded under 1 bar Ar. The potential of zero net current of H_2 oxidation is marked by a dashed vertical line. Data presented are smoothed over every 100 points via the Savitzky-Golay method, as shown in blue and pink solid lines for trace (a) and (b) respectively. Other conditions: scan rate, 10 mV s^{-1} ; pH 7; 25°C ; buffer solution flow rate, 46.2 mL min^{-1} . Panel (b): a difference spectrum triggered by the activation process, with the as-isolated Hyd-2 spectrum as reference.

3.4.3 Summary

This section shows that the strategy of reconstruction of a physiologically-mimicking environment on carbon nanoparticles (with high surface area) by DDAB does improve Hyd-2 loading. Under a low ionic strength condition (15 mM phosphate buffer solution, pH 5.8), the brownish enzyme solution turned transparent after the adsorption process being carried out overnight; approximately 0.3 mg of Hyd-2 was immobilised on 0.1 mg of dry BP2000 particles.

The use of 0.02 mg of BP2000 particles loaded with Hyd-2 (approximately 0.06 mg of Hyd-2) to make a particle electrode is enough to give a good S/N in ATR-IR spectra; cyclic voltammograms collected in the ATR-IR cell by this particle electrode also show large catalytic currents (for both H₂ oxidation and proton reduction) with a negligible overpotential requirement. The high loading is presumably facilitated by hydrophobic interactions, and effective electron transfer can be achieved as the adsorption of Hyd-2 is in the correct orientation. Such a successful design allows more in-depth studies on Hyd-2 chemistry by ATR-IR spectroelectrochemistry, and these will be described in the following Chapters.

Chapter 4: Protein Film Infrared Electrochemistry of *E. coli* Hyd-2

Following the development of carbon particles with high *E. coli* Hyd-2 loading described in Chapter 3, a detailed study on Hyd-2 was performed using protein film infrared electrochemistry (PFIRE). This Chapter represents the first FTIR spectroscopic characterisation of Hyd-2, and describes experiments under turnover and non-turnover conditions.

4.1 Introduction

The redox behaviour of Hyd-2 has not been fully characterised by infrared spectroscopy. The only work reporting FTIR spectra of Hyd-2 is by Sawers and co-workers.¹⁰⁰ They used FTIR spectroscopy to confirm the success of ^{13}C isotope labelling to the active site ligands, and suggested that endogenous cyanide and CO ligands have different metabolic origins. However, they only reported spectra of as-isolated Hyd-2, which appeared to be in the mixture of Ni-A and Ni-B states by comparison to IR data for other hydrogenases.

Thus, in order to perform more in depth studies on the catalytic mechanism and inhibitory effects of small ligands for Hyd-2, assignment of infrared absorption peaks for different redox states is necessary. In this Chapter, acquisition of FTIR spectra with good S/N and under precise potential control was achieved using surfactant-modified BP2000 particles, which have high surface area and high Hyd-2 loading in the correct orientation for effective electron transfer (see Chapter 3).

4.1.1 Redox states

A common picture of what active site states are involved in catalysis for [NiFe]-hydrogenases has emerged through use of different techniques. These states are depicted in Figure 4-1.^{8,55,56} Three redox states, Ni-SI_a, Ni-R and Ni-C, are typically regarded as forming the catalytic cycle (black solid arrows). When H₂ burrows into the active site, it reacts with the EPR-silent Ni-SI_a state, and is immediately polarised and cleaved heterolytically to form a proton and a hydride.¹¹⁶ The hydride is incorporated into the bridging position of the bimetallic centre at the active site to form Ni-R, which is also EPR silent.⁵⁴ The initial proton acceptor upon cleavage of H₂ remains unclear, and thus a generic proton-acceptor, B, is used in Figure 4-1 to describe the cycle. One electron oxidation associated with removal of this proton generates Ni-C, with the hydride retained at the active site. EPR studies showed that the hydride coordinates to the Ni, but in a bridging position with respect to Fe.⁴⁹ Concerted release of an electron and a proton would regenerate the Ni-SI_a state directly from Ni-C, which is ready to restart the catalytic cycle. However, an alternative route for conversion of Ni-C to Ni-SI_a (marked by black dashed arrows) via an intermediate with formal active site oxidation states of Ni^IFe^{II} (known as Ni-L), has been suggested experimentally very recently,^{51,52} although, in most cases, Ni-L is generated following photo-illumination of Ni-C at cryogenic temperatures.⁸ A spectroscopic study of [NiFe]-hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) by Tai *et al.* at cryogenic temperatures suggested that Ni-L is an intermediate state during the conversion of Ni-C to Ni-SI_a; the

proximal $[4\text{Fe}4\text{S}]^{2+/+}$ cluster redox state acts as a gate to control this process,⁵³ as the direct formation of Ni-SI_a from Ni-C can only occur when the proximal cluster is at its oxidised state and is therefore capable of accepting an electron. This viewpoint is further supported by studies on an extreme case: an O₂-tolerant MBH, *E. coli* Hyd-1.^{51,52,60} This type of enzyme is equipped with a unique structure of proximal cluster in a $[4\text{Fe}3\text{S}]$ configuration, which is able to transfer two successive electrons to the active site at high potentials to neutralise reactive oxygen species (*cf.* section 1.6). The midpoint potentials of the $[4\text{Fe}3\text{S}]$ proximal cluster are significantly higher than those for the counterparts in standard hydrogenases (*cf.* Table 1-2), and lead to a separation between electron transfer and proton migration process for the conversion from Ni-C to Ni-SI_a. The Ni-L species is able to be detected by FTIR as a stable state in the dark at room temperature for *E. coli* Hyd-1, and there is a pH-dependent equilibrium between Ni-L and Ni-C for this enzyme; the Ni-L state is favoured at high pH, and Ni-C is dominant at low pH, which means that the extended active site is able to sense changes of external pH.^{51,52}

The reaction of active enzymes with O₂ for standard $[\text{NiFe}]$ -hydrogenases generates at least two most oxidised inactive states (marked with red squares, Figure 4-1), Ni-A and Ni-B, which can be distinguished in electrochemical experiments by the kinetics of their reductive reactivation, and also by the distinct EPR features.^{1,8} The one which can be activated within seconds upon reduction under H₂ is the ready state, Ni-B; a hydroxide ligand has been suggested to be at the bridging position between the Ni^{III} and the Fe^{II} at the

active site.⁸ The species which requires much longer time for reactivation is the unready state, known as Ni-A. The precise chemical identity of the bridging ligand of Ni-A still remains an open question (see section 1.5);⁸ the latest argument on the structure of Ni-A is that it still bears a bridging hydroxide ligand, but one of the bridging thiolate ligands is oxidised to be either sulfenate or sulfenic acid.⁴⁷ The Ni-B state can also be generated from Ni-SI_a by one-electron oxidation, (Figure 4-1, red arrow), although this process is slow without O₂.⁴⁸

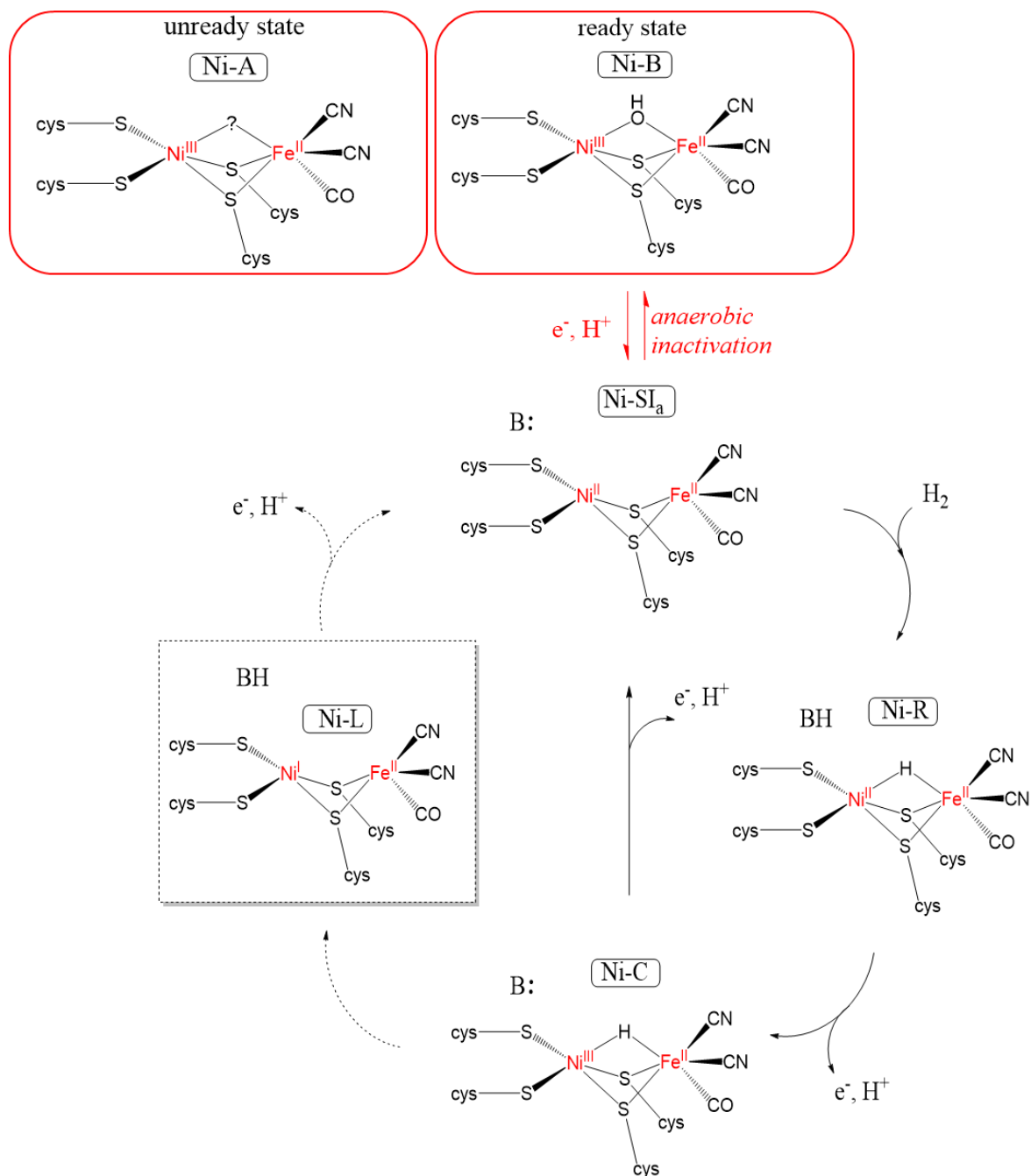


Figure 4-1. Spectroscopically characterised redox states of [NiFe]-hydrogenases. Pathways for H₂ oxidation are shown with black arrows, and the route for reversible anaerobic inactivation is depicted with red arrows. The dashed pathway is discussed in the text. B denotes a generic proton-acceptor. The figure is adapted and reprinted with permission from ref ⁵¹.

4.1.2 FTIR spectroelectrochemical characterisation of

[NiFe]-hydrogenases

FTIR spectroscopy has been a powerful tool for characterisation of hydrogenases, because the set of endogenous diatomic ligands (one CO and two CN⁻ ligands) have vibrational bands in the mid-IR, 2200-1800 cm⁻¹ range, which is clear of absorbances due to water or other biological ligands. By coupling with electrochemistry, the redox states can be identified, since the frequencies of the CO and CN⁻ bands are highly sensitive to changes in coordination and electronic environment at the active site.^{8,46} The classical way for carrying out solution FTIR spectroelectrochemistry is to utilise redox mediators for assistance in electrochemical control over enzyme.⁴⁶ Table 4-1 depicts infrared absorption bands of active site ligands (ν_{CO} and ν_{CN}) of three well-characterised [NiFe]-hydrogenases from different organisms,^{51,53,117–119} for comparison to investigation of Hyd-2 studied in this Chapter.

Overall, in Table 4-1, ν_{CO} and ν_{CN} vary with changing redox level in a similar manner among these three hydrogenases. In terms of absolute values of ν_{CO} and ν_{CN} , there is close similarity between *E. coli* Hyd-1 and *A. vinosum* hydrogenase. In contrast, absolute values for (in terms of ν_{CO}) the hydrogenase from *DvMF* are shifted systematically to higher wavenumbers (by *ca.* 10 cm⁻¹) in comparison. Such a difference suggests the existence of some variation between the enzymes for fine-tuning of their electronic environment,

presumably caused by differences in amino acids surrounding the active site and in electron occupancy of the proximal cluster.

The band positions (ν_{CO} and ν_{CN}) of *E. coli* Hyd-1 listed in Table 4-1 were used for demonstration of how these bands vary in response to changes of redox level of the active site.⁵¹ One electron reduction of the most oxidised state, Ni-B, leads to the formation of Ni-SI_a. Band positions of the diatomic ligands are shifted to lower wavenumbers: ν_{CO} at 1929 cm⁻¹ in addition to symmetric and asymmetric stretches of the two cyanide ligands at 2076 cm⁻¹ and 2085 cm⁻¹, relative to Ni-B (ν_{CO} : 1943 cm⁻¹, symmetric ν_{CN} : 2081 cm⁻¹ and asymmetric ν_{CN} : 2099 cm⁻¹); *cf.* Chatt-Dewar-Duncanson model, Chapter 2, Figure 2-14. The vibration of CO ligand (ν_{CO}) is noted to be more sensitive than ν_{CN} to changes in electron density at the active site: a downward shift by 14 cm⁻¹ in ν_{CO} is seen when the Ni-B state converts to Ni-SI_a, whereas there is only 5 cm⁻¹ difference in symmetric ν_{CN} between these two states. This is because CO is a better π -acceptor ligand and the vibrational frequency of CO is not influenced by the surrounding hydrogen bonding interactions. This means that ν_{CO} features are of greater benefit in identifying redox states and new states of the hydrogenase active site. Therefore, the band positions of ν_{CO} are exploited throughout this Thesis to characterise different redox states and species. The Ni-C state is formed following another electron reduction of Ni-SI_a. Although this state is two electrons reduced from Ni-B, ν_{CO} is shifted to higher wavenumber (1951 cm⁻¹) compared with that of Ni-SI_a (1929 cm⁻¹) and Ni-B (1943 cm⁻¹). This is attributed to the fact that the active site retains a bridging hydride between

the two metals,⁴⁹ and thus the electron density is shifted away from the Ni, ending up with a higher formal charge in the form of Ni^{III}(H⁻). One more electron reduction of Ni-C gives rise to the most reduced state, Ni-R. The Ni-R state is also proposed to bear a bridging hydride in the bimetallic centre,⁵⁴ and is three electrons reduced from Ni-B, with a formal charge of II on the Ni. Thus, in Ni-R, vibrational bands of ν_{CO} and ν_{CN} are all shifted to lower wavenumber, i.e. ν_{CO} : ranging from 1910-1925 cm⁻¹, compared to that of the Ni-C state, i.e. ν_{CO} : 1951 cm⁻¹. There are several different ν_{CO} values associated with Ni-R in Hyd-1, indicating subtle variations in electron density at the Fe, presumably arising from different protonation states of amino acid residues close to the active site. This feature is also observed in other [NiFe]-hydrogenases.⁴⁶ Two forms of Ni-L (also suggesting several protonation sites at the nearby residues) at the Ni^IFe^{II} redox level are characterised by several species with unusually low wavenumber of ν_{CO} and ν_{CN} bands, i.e. 1860-1900 cm⁻¹ of ν_{CO} .

Table 4-1. Values (in cm^{-1}) of ν_{CO} and ν_{CN} for selected states observed in FTIR spectroelectrochemistry of different [NiFe]-hydrogenases.^{a,b,c}

Redox State	<i>A. vinosum</i>		<i>E. coli</i> Hyd-1		<i>D. vulgaris</i> M F	
	$\nu(\text{CO})$	$\nu(\text{CN})$	$\nu(\text{CO})$	$\nu(\text{CN})$	$\nu(\text{CO})$	$\nu(\text{CN})$
Ni-A	1945	2082, 2093	nd	nd	1956	2084, 2094
Ni-B	1943	2079, 2090	1943	2081, 2099	1955	2081, 2090
Ni-SI _a	1931	2073, 2084	1929	2076, 2085	1943	2075, 2086
Ni-C	1951	2073, 2085	1951	2078, 2088	1961	2074, 2085
Ni-L _I	1898	2044, 2060	nd	nd	1911	2048, 2063
Ni-L _{II}	nd	nd	1877	2034, 2051	nd	nd
Ni-L _{III}	nd	nd	1866	2028, 2046	nd	nd
Ni-R _I	1936	2059, 2072	nd	nd	1948	2061, 2074
Ni-R _{II}	1921	2048, 2064	1922	2053, 2070	1932	2052, 2066
Ni-R _{III}	1913	2043, 2058	1914	2049, 2066	1919	2050, 2065
Reference		117,118		51		53,119

^a. *nd* = not determined. ^b. Values shown in bold refer to bands observed at cryogenic temperatures following photo-illumination. ^c. *A. v.* and *DvMF* hydrogenases were characterised by solution FTIR spectroelectrochemistry, whereas *E. coli* Hyd-1 was characterised by PFIRE at a particle electrode.

4.1.3 Hyd-1 and Hyd-2 from *E. coli*

Hyd-1 and Hyd-2 are both native enzymes from *E. coli*. Hyd-1 is an O₂-tolerant MBH, whereas Hyd-2 behaves like a standard hydrogenase, i.e. it is strongly inactivated in the presence of O₂ (*cf.* section 1.6 and 1.7).⁶⁷ Therefore, studies of their responses to the substrate, H₂ (this Chapter), and a series of π -acid ligands, such as CN⁻, CO and isocyanides (see the following Chapters), serve as a good paradigm for the two types of [NiFe]-hydrogenases. Figure 4-2 shows typical features observed for them in electrochemistry. Hyd-2 is a good bidirectional hydrogenase; a negligible overpotential is required for H₂

oxidation and a significant proton reduction current is observed even under 1 bar H_2 without suffering from severe product inhibition. In contrast, a more oxidising condition is needed for Hyd-1 to catalyse H_2 oxidation, and it is unable to function in reverse under these conditions (1 bar H_2 , pH 6).

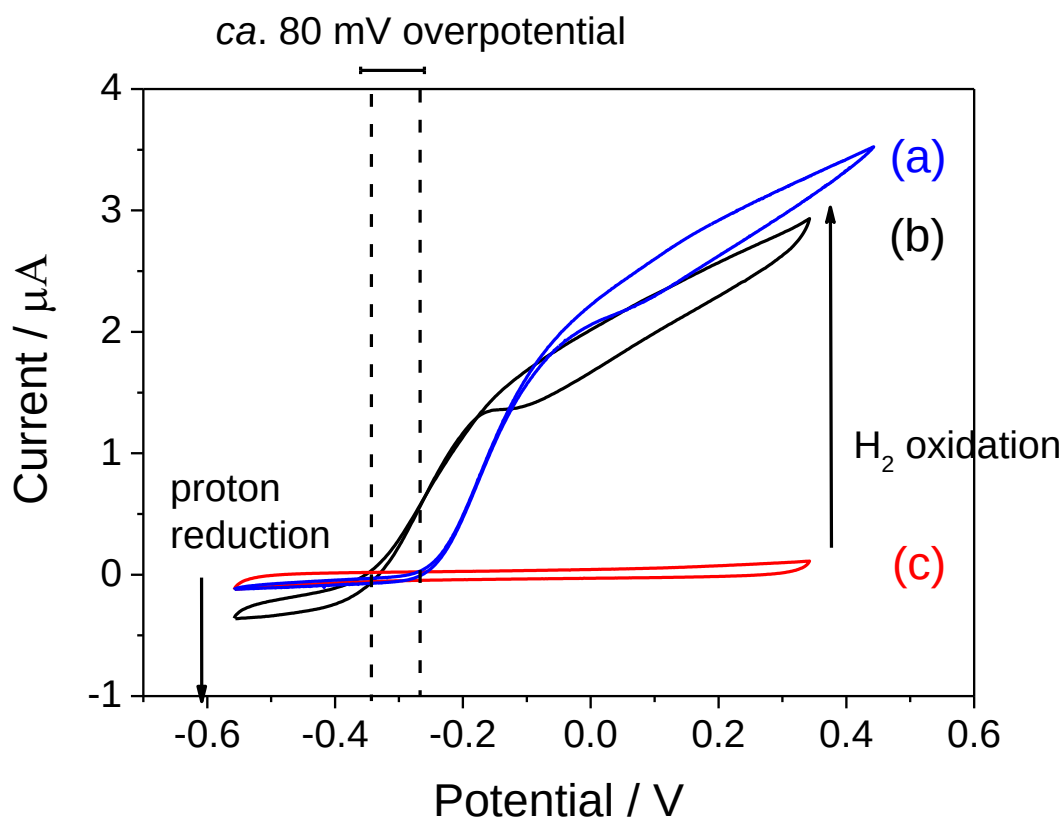


Figure 4-2. Cyclic voltammograms for Hyd-1 (a) and Hyd-2 (b) directly adsorbed on a bare PGE electrode under 1 bar H_2 . Trace c shows the charging current of the PGE electrode recorded prior to the measurements. Other conditions: pH 6; 28 °C; electrode rotation rate, 2000 rpm; scan rate, 10 mV s⁻¹.

4.2 Experimental

Infrared spectroelectrochemistry experiments were performed inside an anaerobic glovebox (<1 ppm of O₂, Glove Box Technology Ltd) at a working electrode comprising Hyd-2 immobilised on DDAB-modified BP2000 on a silicon multibounce ATR prism (8.39×5×1 mm³ with an incidence angle of 39°, Crystal GmbH). A miniature SCE reference electrode, constructed in-house, and a Pt wire counter electrode were inserted into the solution compartment of the cell. A potassium phosphate buffer solution (15 mM, pH 5.8) was used for particle preparations throughout. A stock dispersion of BP2000 particles (Cabot Corp., 20 mg mL⁻¹) was prepared by ultrasonication (60 min). DDAB-modified BP2000 particles were then prepared by mixing 5 µL aliquots of DDAB solution (10 mM) with 5 µL aliquots of well-dispersed BP2000 suspension (20 mg mL⁻¹). The mixture was left at 4°C overnight; removal of excess DDAB was then carried out by centrifuging particles to take out the supernatant. The particles were then washed with 100 µL aliquots of buffer solution (pH 5.8) and collected by centrifugation (this process was repeated for three times) before further use for Hyd-2 adsorption.

Escherichia coli hydrogenase 2 (Hyd-2) was prepared according to a published procedure and concentrated to 1.5 mg mL⁻¹.⁶⁷ A 200 µL aliquot of Hyd-2 solution (pH 5.8) was mixed with 5 µL aliquots of the DDAB-modified BP2000 (20 mg mL⁻¹), and was left at 4 °C overnight for adsorption. Removal of unadsorbed Hyd-2 was carried out by centrifuging particles to take out the

supernatant. The particles were then washed to give a final dispersion, also approximately 20 mg mL⁻¹. A 1 µL aliquot of this particle suspension was then deposited on the silicon IRE and allowed to dry partially before assembling the cell on the top.

Spectroelectrochemistry was carried out using a custom-modified ATR-IR accessory (GladiATR, PIKE Technologies) and a Varian 680-IR spectrometer. A mixed buffer solution (pH 6) was used throughout this Chapter. Data collection and analysis were performed using Varian Resolutions Pro 4.0 software. Spectra were recorded at 4 cm⁻¹ resolution and are reported as an average of 1000 scans. Electrochemical control was provided by an Autolab PGSTAT 128N potentiostat manipulated by Nova software (EcoChemie). Potentials are quoted with respect to SHE using the correction $E_{\text{SHE}} = E_{\text{SCE}} + 242 \text{ mV}$ at 25 °C.

4.3 Results and discussion

4.3.1 Overview of Hyd-2 under turnover and non-turnover conditions

Results

Figure 4-3 shows spectra of the ν_{CO} region recorded at selected electrode potentials, under 1 bar Ar or H₂ at pH 6, on a single sample of immobilised Hyd-2 on surfactant-modified particles, together with the corresponding current-time trace. The adsorbed enzyme was first activated at -0.6 V under H₂ prior to the experiments. At the lowest potential applied, -0.594 V, a large

electrocatalytic proton reduction current is observed, under either Ar or H₂. A comparison of the reduction currents recorded in the ATR-IR cell under these conditions reveals that H₂ is a weak product inhibitor of proton reduction by Hyd-2, i.e. *ca.* -50 μ A under Ar and *ca.* -25 μ A under H₂. The observation is consistent with the inherent property of Hyd-2: *a good bidirectional hydrogenase* (at least *in vitro*) revealed by previous electrochemical studies.⁶⁷

Under a H₂ atmosphere, at potentials above the formal reduction potential of the H⁺/H₂ couple, -0.353 V (calculated by the Nernst equation under these conditions: 1 bar H₂; pH 6; 25°C), the current increases significantly as the electrochemical driving force is raised for H₂ oxidation (Figure 4-3 a, red). The oxidation current is extremely stable at -0.324 V, -0.199 V and -0.074 V over 1200 s, indicating that the high flow rate of H₂-saturated buffer solution through the cell (46 mL min⁻¹) provides effective mass transport of H₂, and that the enzyme is adsorbed robustly on the surfactant-modified carbon. No enhancement in current occurred as the flow rate of H₂-saturated buffer was increased beyond 34 mL min⁻¹. At the most positive potential applied (+0.356 V), the current decays slightly over time, consistent with the well-established oxidative inactivation of [NiFe]-hydrogenases, which is a slow process under anaerobic conditions and in the presence of H₂.⁴⁸

The ν_{CO} bands observed in spectra at different potentials under Ar (Figure 4-3, panel b) reveal a set of active site states for Hyd-2. At the most positive potential applied (+0.356 V), three bands at 1957, 1948 and 1941 cm⁻¹ are

detected. As the potential is taken more negative to -0.074 V and then -0.199 V, the population of band at 1957 cm^{-1} decreases, associated with an increase of a band at 1946 cm^{-1} . At potentials where proton reduction occurs (-0.324 V, -0.414 V and -0.594 V), two more peaks at 1964 and 1954 cm^{-1} appear. The population of band at 1946 cm^{-1} decreases significantly at these potentials for proton reduction. In sharp contrast, the population of bands at 1948 and 1941 cm^{-1} remains nearly the same at all applied potentials, as suggested by spectral deconvolution and Gaussian peak fitting. The fact that the intensity of these two peaks are not changed in response to application of potentials is further confirmed by the difference spectra (Figure 4-4), which are calculated from absolute spectra of panel b in Figure 4-3. The difference spectrum depicted in panel a (Figure 4-4) is obtained by the absolute spectrum collected at +0.356 V minus the one recorded at -0.074 V. It clearly shows a sharp positive band at 1957 cm^{-1} and a negative peak at 1946 cm^{-1} . The other spectrum shown in panel b (Figure 4-4) is generated by “-0.199 V minus -0.594 V”, which shows a sharp peak at 1946 cm^{-1} in the positive direction and two negative bands at 1954 and 1964 cm^{-1} . The peaks at 1948 and 1941 cm^{-1} with concentrations staying almost constant are present in the as-isolated Hyd-2 adsorbed on surfactant-modified particles (Figure 4-4, panel c).

Overall, under 1 bar Ar, the ν_{CO} band at 1957 cm^{-1} present at the most positive potential applied, +0.356 V, is assigned to the Ni-B state. The possibility of forming Ni-A under these conditions is ruled out, since this state is generally thought to be generated only when O_2 is present.^{1,8} All of the experiments were

carried out under anaerobic conditions inside gloveboxes, including sample preparations, and FTIR measurements; the gases used in experiments were also passed through an O₂-purifier to ensure having an O₂-free environment. As the potential is taken more negative to -0.074 V and then -0.199 V, growth of a band at 1946 cm⁻¹ is assigned to Ni-SI_a. The peak at 1964 and 1954 cm⁻¹ appearing during proton reduction at -0.414 and -0.594 V are assigned to the Ni-C and Ni-R state, respectively. The set of active site states of Hyd-2 displays close similarity to that of hydrogenase from *DvMF* observed in solution FTIR spectroscopy (*cf.* Table 4-2).¹¹⁹ This feature implies that these two enzymes share a similar electronic environment within the active site, and thus information from *DvMF* hydrogenase serves as a valuable reference point for assistance in redox states identification of Hyd-2.

Figure 4-3, panel c shows spectra recorded at the same potentials, but under 1 bar H₂. At potentials involving proton reduction (-0.594 V and -0.414 V), the spectra collected under both Ar and H₂ are very similar; Ni-C, Ni-R and Ni-SI_a are always present, although the concentration of each species is different between under Ar and under H₂. The population of the Ni-C state under Ar is greater than that under H₂, and Ni-R is present at slightly lower concentration under Ar. This is consistent with the higher rate of proton reduction catalysis under Ar that keeps the enzyme more oxidised.

At more positive potentials where Hyd-2 engages in electrocatalysis of H₂ oxidation, there are significant differences in state distributions. During H₂ oxidation, Ni-C persists as the majority species at all applied potentials,

together with a significant proportion of Ni-SI_a and Ni-R, which are now observed even at +0.356 V where they are absent under an inert atmosphere. Also, under H₂, the Ni-SI_a state is observed in a lower concentration at -0.199 V and persists up to the highest potential (with concentration increasing slightly as potential steps to more positive values), and the onset potential for formation of Ni-B is much more positive; only a small proportion of Ni-B is present at +0.356 V.

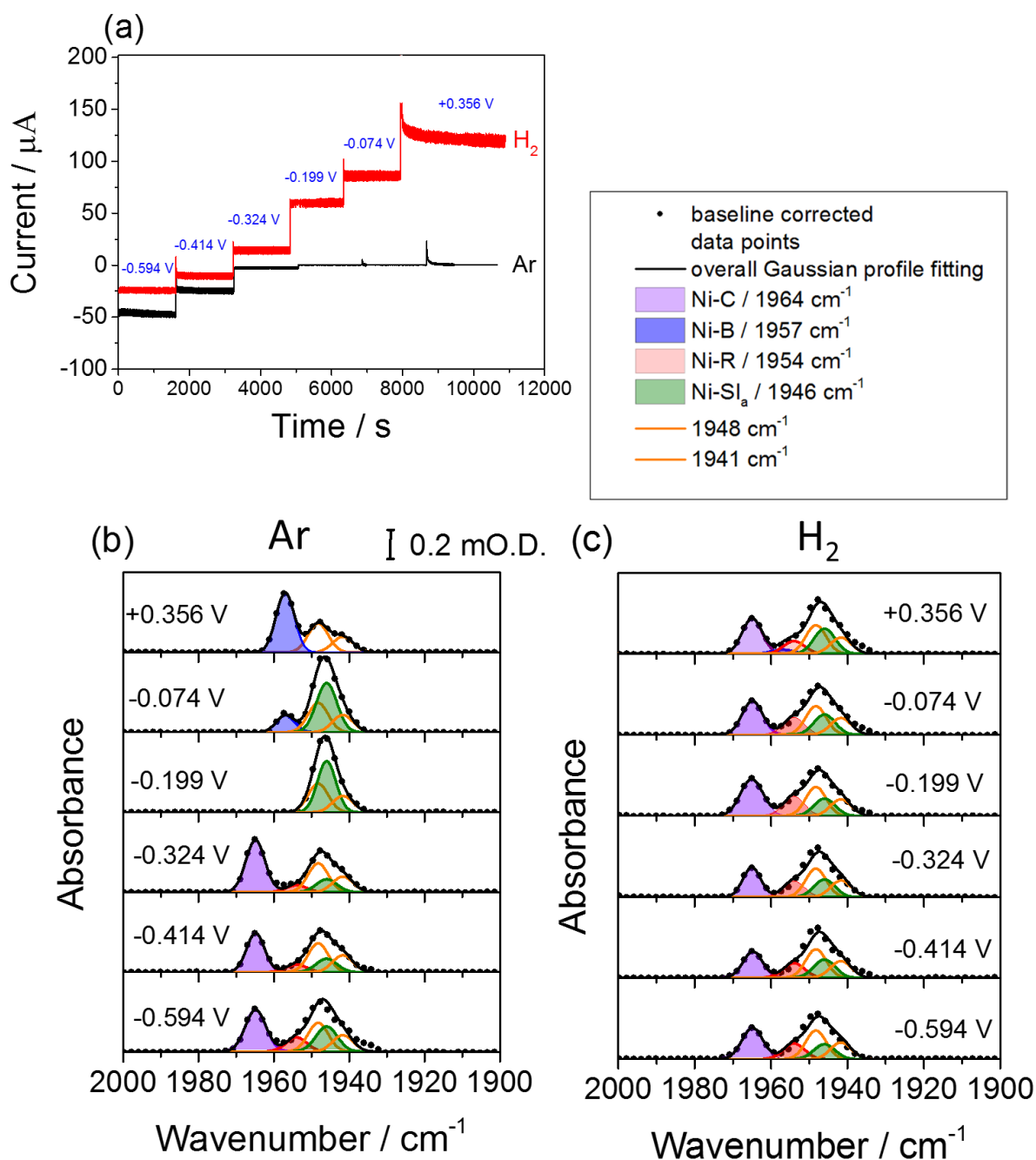


Figure 4-3. (a) Current-time traces of Hyd-2 collected in the ATR-IR cell in Ar-saturated (black) and H_2 -saturated (red) buffer, pH 6; (b),(c) absolute spectra showing the ν_{CO} region at each potential of Hyd-2 under Ar (b) and H_2 (c).

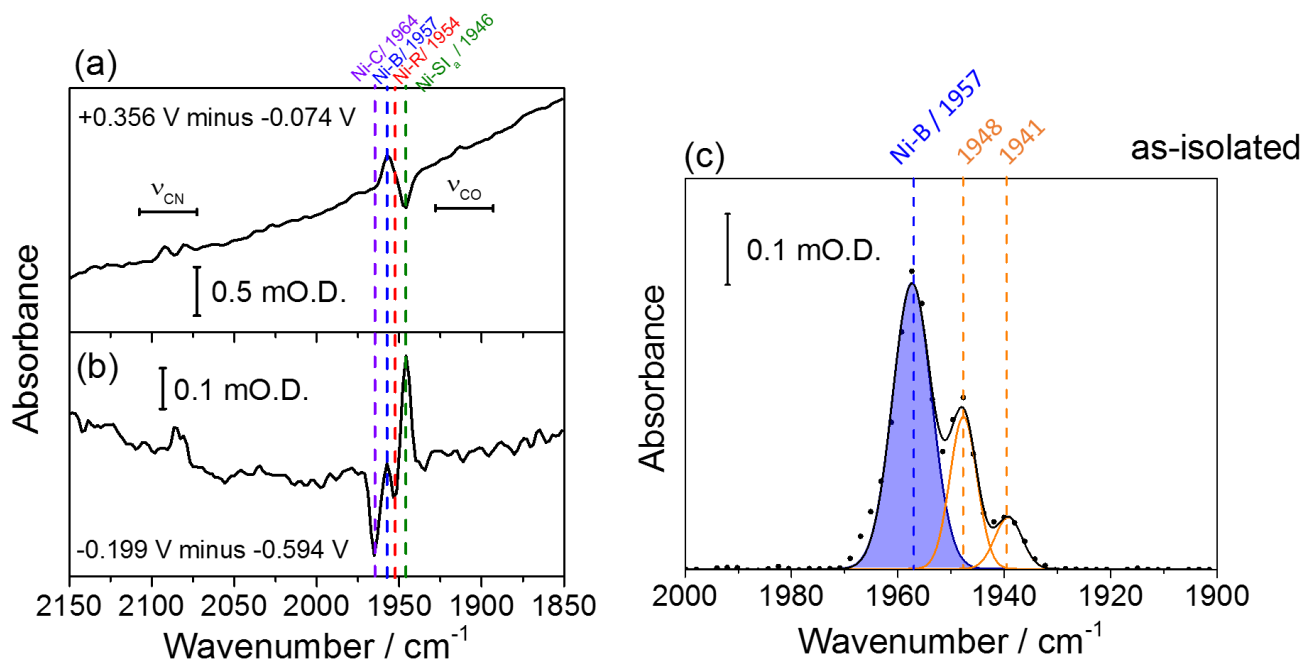


Figure 4-4. Difference spectra of Hyd-2 calculated from data under Ar shown in Figure 4-3. Panel a: +0.356 V minus -0.074 V. Panel b: -0.199 V minus -0.594 V. Panel c: an absolute spectrum of as-isolated Hyd-2 immobilised on surfactant-modified particles under Ar.

Table 4-2. Values (in cm^{-1}) of ν_{CO} and ν_{CN} of redox states observed in FTIR spectroscopy for *E. coli* Hyd-2 (by PFIRE) and *Dv*MF [NiFe]-hydrogenase in solution.

Redox State	<i>E. coli</i> Hyd-2		<i>D. vulgaris</i> M F	
	$\nu(\text{CO})$	$\nu(\text{CN})$	$\nu(\text{CO})$	$\nu(\text{CN})$
Ni-B	1957	2081, 2092	1955	2081, 2090
Ni-SI _a	1946	2075, 2086	1943	2075, 2086
Ni-C	1964	nd	1961	2074, 2085
Ni-R _I	1954	2060, nd	1948	2061, 2074
Ni-R _{II}	nd	nd	1932	2052, 2066
Ni-R _{III}	nd	nd	1919	2050, 2065
reference	<i>current work</i>		119	

The changes of redox states are reversible with potential (Figure 4-5, panel a); stepping potential down to -0.594 V under Ar (after the experiment carried out in the oxidative direction, i.e. from low to high potential, presented in Figure 4-3, panel b), led to a sharp negative peak at 1957 cm^{-1} (ν_{CO} , Ni-B) and a positive band at 1964 cm^{-1} (ν_{CO} , Ni-C) in the difference spectrum; the spectrum collected at +0.356 V in the former experiment was used as reference. The adsorbed enzyme sample is very stable; after 36 h of experiments at room temperature, there is no detectable change in amide bands of the protein (1652 cm^{-1} and 1545 cm^{-1} , Figure 4-5, panel b). Also, the active site peaks (ν_{CO}) are still clearly visible in the absolute spectrum (without baseline correction) after 36 hours of experimentation (Figure 4-5, panel c).

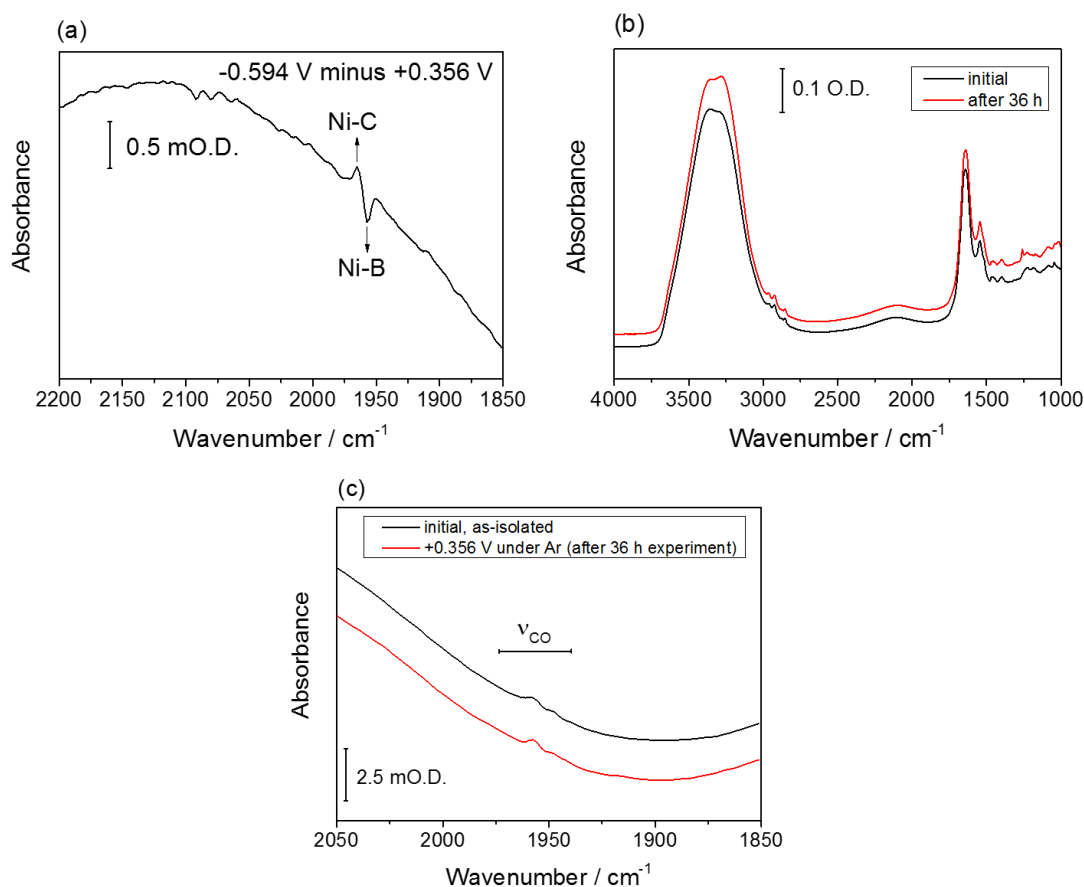


Figure 4-5. Panel a: a difference spectrum of Hyd-2 calculated by “ -0.594 V minus $+0.356 \text{ V}$ ” under Ar, showing the active site states are reversible with potential. Panel b: absolute spectra of adsorbed Hyd-2. Black: as-isolated Hyd-2 at the beginning of experiments. Red: at the end of experiments at $+0.356 \text{ V}$; approximately 36 hours later. Panel c: an expanded view of ν_{CO} region of absolute spectra in panel b.

Discussion

The absolute values of ν_{CO} of active site states of *E. coli* Hyd-2 are systematically higher by *ca.* 10 cm^{-1} compared with those for *E. coli* Hyd-1. This reveals that the electronic environments of the active sites are different between these two enzymes, presumably due to variations in amino acids close to the active site of the protein and in electron occupancy at the proximal FeS cluster. The origin of the presence of species (with absorption bands at 1941 and 1948 cm^{-1}) which do not respond to redox changes is unclear. These species might be present in the as-isolated enzyme solution, or due to damage of the protein upon immobilisation on the carbon. Since the presence of these species does not affect the catalytic behaviour of Hyd-2 and data interpretation in FTIR spectroscopy, there is no further investigation of them tentatively.

The differences between the Ar and H_2 data at potentials involving H_2 oxidation can be explained by reference to the catalytic cycle shown in Figure 4-1 (black solid arrows, see section 4.1.1): *H₂ attacks Ni-SI_a to form Ni-R immediately, and regeneration of the Ni-SI_a state proceeds via Ni-C to restart the catalytic cycle.* At intermediate potentials under H_2 (-0.199 V and -0.074 V, Figure 4-3, panel c), a distribution of states of Ni-R, Ni-C and Ni-SI_a is detected led by fast attack of H_2 on Ni-SI_a. At the highest potential (+0.356 V), a small amount of Ni-B is present, contributed by the oxidative inactivation. The observation of Ni-C, Ni-R, and Ni-SI_a under H_2 at +0.356 V, which is outside the potential window where they are observed under non-turnover conditions,

provides strong evidence that these three states are generated in response to catalytic H₂ oxidation.

The validity of applying microscopic reversibility into mechanistic proposals for H₂ cycling catalysed by hydrogenases has been challenged, because the external driving force for the reactions are thought to be different.¹²⁰ However, a distribution of the same states of Ni-R, Ni-SI_a, and Ni-C is also observed at low potentials (-0.594 V and -0.414 V) where Hyd-2 participates in electrocatalysis of proton reduction (Figure 4-3). This implies that the proton reduction might be conducted via the same elementary processes as H₂ oxidation, but in a reverse direction, i.e. protons react with the Ni-R state to form Ni-SI_a, and the regeneration of Ni-R for the next catalytic cycle proceeds via Ni-C, which is one electron reduced from Ni-SI_a. Or, the precise detail of the mechanism might be different for proton reduction, but the principal 'equilibrium' states involved are the same as H₂ oxidation.

4.3.2 Differences between Hyd-1 and Hyd-2 in response to catalytic turnover

*Results of Hyd-1 (data are provided by Ricardo Hidalgo)*⁵¹

Figure 4-6 depicts a set of data of Hyd-1 together with the corresponding current-time trace. The experiments were conducted under comparable conditions to the Hyd-2 data depicted in Figure 4-3. In contrast to Hyd-2, at the most negative potential applied, -0.594 V, there is no detectable electrocatalytic current under either Ar or H₂ (panel a), consistent with the poor ability of Hyd-1 to catalyse H₂ production, at pH 6 (see section 1.6).^{66,67} Under a H₂ atmosphere, the current increases significantly as the potential is stepped to more positive values, namely the electrochemical driving force is raised, (panel a, black). At the most positive potential applied (+0.356 V), the slow process of oxidative inactivation occurs, revealed by the fact that the oxidation current decays slightly over time.

The spectra collected at different potentials under Ar (panel b) reveal a set of active site states for Hyd-1 (see Table 4-1 for details of peak positions and assignments). The species of Ni-R dominates at the most negative potential applied (-0.594 V), and it exists in two forms indicated by two ν_{CO} bands at 1914 and 1922 cm⁻¹ that are always present in the same ratio of intensities, which is presumably contributed by different protonation states within the extended active site region. As the potential is stepped more positive to -0.199 V, the concentration of the Ni-R species decreases, associated with an increase of

three species: Ni-C, Ni-SI_a, and Ni-L. The populations of Ni-L, Ni-C, and Ni-R decrease as the potential is stepped up to -0.074 V, where the Ni-SI_a state is the dominant species. At the most positive potential applied (+0.356 V), Ni-B is the only state detected, showing that the whole enzyme sample has responded to application of the potential. Spectra collected at the same potentials but under 1 bar H₂ are shown in panel c. At -0.594 V, where negligible catalytic turnover, i.e. proton reduction, is detected, there are no obvious differences to the spectrum under Ar. However, at more positive potentials where Hyd-1 participates in electrocatalytic H₂ oxidation, there are considerable differences in the distribution of states. When the enzyme is turning over under H₂, Ni-R persists as the majority species at all applied potentials, together with a significant proportion of Ni-C and Ni-L. Also, under H₂, Ni-SI_a is observed in a lower concentration at -0.074 V and persists up to the highest potential, and the onset potential for formation of Ni-B is also more positive. The most striking difference between the Ar and the H₂ data occurs at +0.356 V, where states of Ni-SI_a, Ni-C, Ni-L and Ni-R are all present; this potential is outside the potential window where these species are observed under non-turnover conditions. This feature provides strong evidence that these states are generated in response to catalytic H₂ oxidation, in which the concentrations of Ni-C and the Ni-L states are found to remain in the same ratio at all potentials applied for Hyd-1.

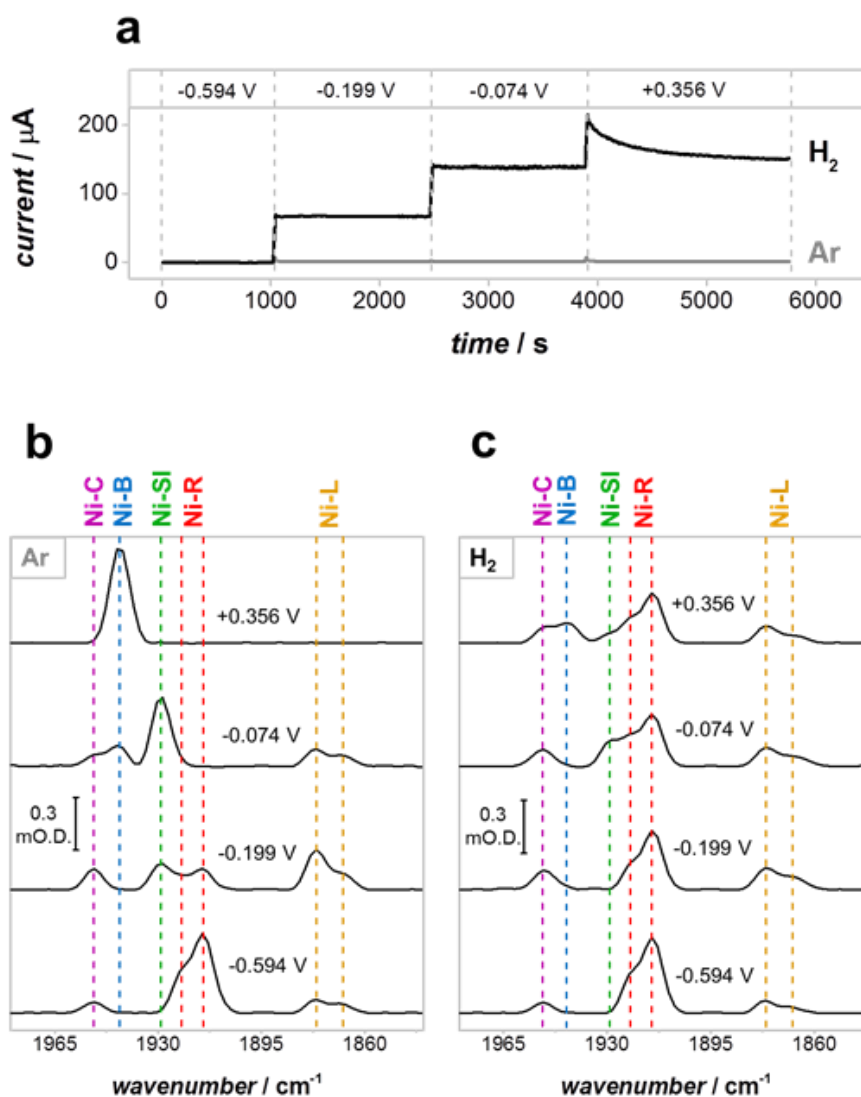


Figure 4-6. Panel (a): current-time traces of Hyd-1 collected in the ATR-IR cell in Ar-saturated (grey) and H_2 -saturated (black) buffer, pH 6; Panel (b),(c): absolute spectra showing the ν_{CO} region at each potential of Hyd-1 under Ar (b) and H_2 (c). Figures are adapted and reprinted with permission from ref ⁵¹.

Discussion

The differences between the Ar and the H₂ data of Hyd-1 can be explained by reference to the catalytic cycle involving Ni-SI_a, Ni-R, Ni-C, and Ni-L states suggested from previous studies (Figure 4-1, black dashed arrows).^{51–53} When H₂ burrows into the active site, the heterolytic cleavage of H₂ happens quickly upon the substrate encountering the active site at the Ni-SI_a level, which has an open bridge to proceed catalysis. The Ni-R state forms rapidly following the attack of H₂. However, the route for regeneration of the Ni-SI_a state to restart the catalytic cycle is different in Hyd-1 compared to Hyd-2; for Hyd-1, the proton retained at the bridging position of Ni-C starts to migrate to the nearby base ahead of electron transfer, leading to the Ni-L species with an open bridge at a Ni^IFe^{II} redox level. This step is followed by one-electron reduction of Ni-L and loss of the proton to re-form Ni-SI_a.

The Ni-L state has been considered as an artefact without being relevant to the catalytic cycle for a long time. This is because it is normally generated under special conditions, i.e. following photo-illumination of Ni-C at cryogenic temperatures.⁸ Thus, it is worth considering why the Ni-L state is so easily observed in Hyd-1 and is likely to be a true intermediate during catalytic turnover. A previous work from Tai *et al.* suggested that the proximal cluster plays an important role in gating the formation of Ni-SI_a, which is only able to be generated from Ni-C when the proximal cluster is at its oxidised state, i.e. with the ability to accept an electron.⁵³ This argument is fully consistent with observations in Hyd-1. Hyd-1, as an O₂-tolerant MBH, it is equipped with an

unusual proximal cluster in a [4Fe3S] configuration, which is capable of transferring two successive electrons to the active site at high potentials to prevent the formation of the unready state upon encountering O₂ (see section 1.6).^{57,60} Moreover, the redox potentials of the medial and proximal clusters are not only significantly higher than for their counterparts in O₂-sensitive hydrogenases, but also are considerably more positive than the Ni-C/Ni-SI_a redox potential under any pH conditions under an inert atmosphere.^{52,60} Therefore, the elementary electron transfer step that converts Ni-C to Ni-SI_a is readily impeded, because the immediate electron acceptor sites are pretty much always fully occupied. This results in formation of the kinetically preferred product, Ni-L, which can be detected all the time at intermediate potentials under Ar and is generated in response to the catalytic turnover. In sharp contrast, the oxidation of Ni-C to Ni-SI_a for Hyd-2 (also among other standard hydrogenases) proceeds in a single proton-coupled electron-transfer (PCET) process. This is expected from the relationship of redox potentials between the proximal cluster and the Ni-C/Ni-SI_a redox couple. As mentioned earlier, the redox potentials of all three FeS clusters in standard hydrogenases are considerably more negative than for their counterparts in O₂-tolerant MBHs (*cf.* Table 1-2), and thus they are closer to the redox potential of the Ni-C/Ni-SI_a couple. For example, the apparent redox potential of the proximal cluster for hydrogenase from *DvMF* is < -300 mV (pH 7.7) and is -337 mV (pH 7.8) for the Ni-C/Ni-SI_a couple.^{62,119} Thus, the elementary process of electron

transfer for the formation of Ni-SI_a from Ni-C is able to proceed smoothly due to the compatible energy between these sites.

The distinct redox potentials of FeS clusters between Hyd-1 and Hyd-2 also lead to different distributions of states in response to catalytic turnover. For Hyd-1, Ni-R is always the dominant species, with concentrations far greater than that of Ni-SI_a (see Figure 4-6). This is because the redox potentials of the three FeS clusters are significantly high,⁶⁰ they are far more likely to be in the reduced states any time when H₂ is turning over, which is the prerequisite for formation of the most reduced state, Ni-R. In sharp contrast, due to the similar redox potentials between the proximal FeS cluster and the Ni-C/Ni-SI_a couple for standard [NiFe]-hydrogenases, the concentration of Ni-SI_a is greater or similar to that of Ni-R for Hyd-2 when the substrates are turning over (see Figure 4-3). These different features between Hyd-1 and Hyd-2 in distribution of states during catalytic turnover also result in their distinct responses to CO inhibition (see Chapter 6).

In summary, the striking difference in distribution of states during catalytic turnover between Hyd-1 and Hyd-2 is governed by the distinct redox potentials of the FeS clusters. This factor leads to distinct kinetics in the elementary process for the conversion from Ni-C to Ni-SI_a; a “concerted” proton and electron transfer step for Hyd-2, and a “sequential” proton and electron transfer steps for Hyd-1.

Chapter 5: Infrared Spectroelectrochemical Study of Cyanide Inhibition of [NiFe]-Hydrogenases from *Escherichia coli*

This chapter presents results of cyanide inhibition of *E. coli* Hyd-1 and Hyd-2, and shows an unusual reaction of [NiFe]-hydrogenases with cyanide; cyanide enhances the rate largely for oxidative formation of the ready state, Ni-B, revealed by PFE, PFIRE and EPR studies.

5.1 Introduction

This project, conducted in collaboration with Suzannah Hexter and Fraser Armstrong, examines how a common inhibitor of metalloenzymes, cyanide, reacts with [NiFe]-hydrogenases from *E. coli*.⁸³ Only a few studies have been reported on cyanide inhibition of [NiFe]-hydrogenases. The first observation of this effect comes from *Azotobacter vinelandii*, in which cyanide was either applied to whole cell samples or to cell-free extracts.^{80,81} The results showed that cyanide acts as a strong inhibitor of H₂ oxidation activity when O₂ is present but has much less effect when cyanide is incubated with reduced, active hydrogenase under H₂. Further studies via spectrophotometric assay methods on purified hydrogenase of *A. vinelandii* showed that cyanide causes irreversible inhibition of enzyme at its oxidised state.⁸²

More detailed studies were carried out by Suzannah Hexter by PFE on an electrode-immobilised films of *E. coli* Hyd-1 and Hyd-2 (Figure 5-1). Cyclic voltammetry examines how cyanide effects the activities of both enzymes over a potential range of 810 mV. Introducing cyanide in contact with Hyd-2 (panel a) has no effect on the H⁺ reduction or H₂ oxidation activity below *ca.* -200 mV. However, as the potential is swept to more positive values, the presence of cyanide results in a significant decrease in current compared to when no cyanide is present. The inactivation that is demonstrated by the drop in current continues throughout the cyclic voltammogram until a certain potential is reached in the return scan (approximately -120 mV), at which point

Chapter 5. Cyanide inhibition of [NiFe]-hydrogenases

reactivation of the enzyme commences, as seen by a sharp increase in current until the activity of the enzyme returns to its uninhibited value, eventually. This inhibitory effect is not specific to Hyd-2; Hyd-1 shares a similar feature in an analogous experiment (panel b). Compared with Hyd-2, a higher potential is required for cyanide to have an effect on H₂ oxidation for Hyd-1; a decrease in current is observed at potentials higher than -100 mV. The activity is easily restored in the reductive potential sweep; the reactivation occurs at potentials below approximately +160 mV. Further studies by EPR (also conducted by Suzannah Hexter) on Hyd-2 revealed that the final enzyme product when cyanide is present, formed within a second (even under 100% H₂) is the resting state known as Ni-B (containing a bridging hydroxide ligand at the active site), which is normally produced slowly by anaerobic oxidation.⁴⁸

Since the EPR technique is limited to probe species with unpaired electrons, in this Chapter, FTIR spectroelectrochemistry is exploited in an attempt to examine if there is any new species formed regardless of redox and spin state, when cyanide is present.

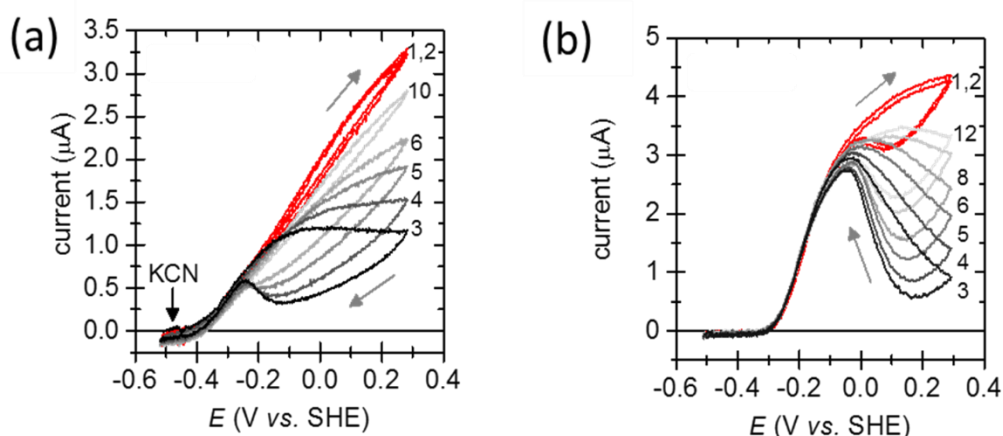


Figure 5-1. Cyclic voltammograms reveal how cyanide effects (a) Hyd-2 and (b) Hyd-1, when it is introduced at -0.52 V during a set of scans. (Scan numbers are indicated alongside each plot.) Scans before cyanide injection are shown in red, and those after are depicted in shades of grey. Diminishing inhibitory effects during successive cycles in both figures are due to removal of cyanide in a gaseous form as HCN, as the cell is not sealed. Other conditions: pH 7; 25 °C; scan rate, 4 mV s⁻¹; ω (electrode rotation rate), 100 rpm; 1 bar H₂. The final concentration of CN⁻ in the cell immediately after the injection was 1.4 mM. Grey arrows indicate the direction of the scan. Results are provided by Suzannah Hexter.⁸³ Figures are adapted and reprinted with permission from ref ⁸³. Copyright © 2014, American Chemical Society

5.2 Hyd-2

5.2.1 ATR-IR spectroelectrochemistry of Hyd-2 at a carbon particle network electrode

When this project was in collaboration with Suzannah Hexter and Fraser Armstrong, and was published in *J. Am. Chem. Soc.* 2014, **136**, 10470–10477,⁸³ solution ATR-IR spectroelectrochemical experiments were carried out by means of a particle network electrode (*cf.* section 3.1.1) to check for the presence of new species regardless of redox and spin state during cyanide reaction. This is because by the time this project was published, the use of surfactant-modified particles for Hyd-2 immobilisation was not developed yet.

Figure 5-2 shows redox-triggered difference spectra (background recorded for Hyd-2 at -0.4 V) recorded in N₂-saturated buffer (pH 7) as the potential was stepped to more positive values in 0.1 V intervals up to 0 V, with and without KCN present. The spectra presented were recorded 20 min after each potential step, at which point the enzyme was found to have reached diffusion-controlled equilibrium with the electrode potential. Only the changes in absorbance attributed to the endogenous CO ligand, ν_{CO} , are well resolved (2000-1900 cm⁻¹ region). The greater intensity of the ν_{CO} peaks compared to those resulting from vibrations of the endogenous CN⁻ ligands (ν_{CN}), 2150 - 2050 cm⁻¹ region, mean that ν_{CO} features are of greater benefit in identifying states and characterising new species.

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As shown in the difference spectra in panel (a), steps from -0.4 V to 0 V resulted in loss of the Ni-C state, and formation of Ni-SI_a, see section 4.2 for detailed characterisation. Panel (b) depicts an analogous set of redox-triggered difference spectra recorded after introducing 10 mM KCN (final concentration) at -0.4 V into the cell solution in contact with the enzyme sample. In the presence of cyanide, a formation of the Ni-B state is now obvious at -0.2 V, accompanied by a weaker band at 1946 cm⁻¹ attributed to the Ni-SI_a state. As the potential is stepped to -0.1 V and then 0 V, the amount of the Ni-B state continues to increase, and the Ni-SI_a state disappears. A weak feature is visible at 1937 cm⁻¹ in the spectra of panel (b), and may correspond to a small portion of the Ni-SI_r state. Thus, the spectra recorded in the presence of cyanide indicate almost complete conversion of Ni-C to Ni-B after 20 min at 0 V, whereas in the analogous experiment conducted in the absence of cyanide (panel a), Ni-C converts only to Ni-SI_a under the same conditions. No additional vco peaks can be identified in the spectra for Hyd-2 in the presence of cyanide, showing that no intermediate in the cyanide reaction is observed.

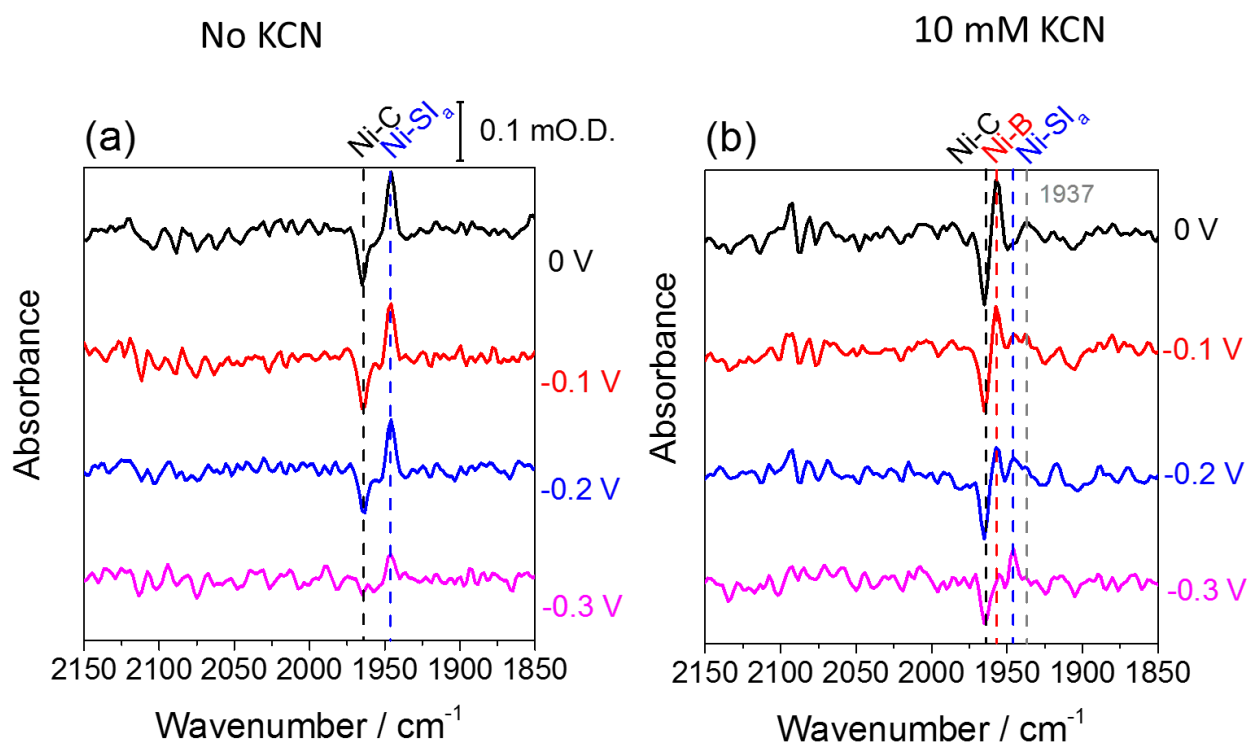


Figure 5-2. Solution ATR-IR redox-triggered difference spectra of Hyd-2 at a carbon particle network electrode, constructed from trapping carbon black particles and enzyme molecules in aqueous Nafion suspension (titrated to neutral pH). Background spectra were collected at -0.4 V, followed by potential steps in 0.1 V intervals. Each spectrum was recorded 20 min after the potential step: (a) without cyanide present; and (b) with 10 mM cyanide present. All spectra were recorded in N₂-saturated buffer (pH 7).

5.2.2 PFIRE of Hyd-2

As the point addressed in section 3.1.1, the oxidative inactivation state, Ni-B, is expected to be present at high potentials, such as 0 V, revealed from PFE (*cf.* Figure 2-4). However, a formation of the Ni-B state is not observed in solution ATR-IR spectroscopy at a particle network electrode when cyanide is absent (*vide supra*). Hence, once adsorption of Hyd-2 on BP2000 could be achieved to a high loading for spectroscopic detection under direct electrochemical control, an analogous set of experiments were carried out to re-examine the cyanide reaction (Figure 5-3). In sharp contrast to the ATR-IR spectra recorded at the particle network electrode (Figure 5-2, panel a), the Ni-B state appears at -0.1 V without cyanide present (Figure 5-3, panel a). In the presence of 10 mM cyanide (Figure 5-3, panel b), the Ni-B state is obvious at a lower potential, -0.2 V, accompanied by a similar intensity band at 1946 cm^{-1} attributed to Ni-SI_a. As the potential is stepped to -0.1 V, the Ni-B state continues to grow, and the Ni-SI_a state disappears. Thus, the spectra recorded in the presence of cyanide indicate complete conversion of Ni-C to Ni-B after 20 min at -0.1 V. In contrast, for the experiment conducted in the absence of cyanide (Figure 5-3, panel a) under comparable conditions, Ni-C converts only to Ni-SI_a at -0.2 V, and there is still a small proportion of Ni-SI_a at -0.1 V. The results share the same feature with solution ATR-IR at a carbon particle network electrode: *Ni-B is promoted to be formed in the presence of 10 mM KCN, and no new species are observed during the cyanide reaction.*

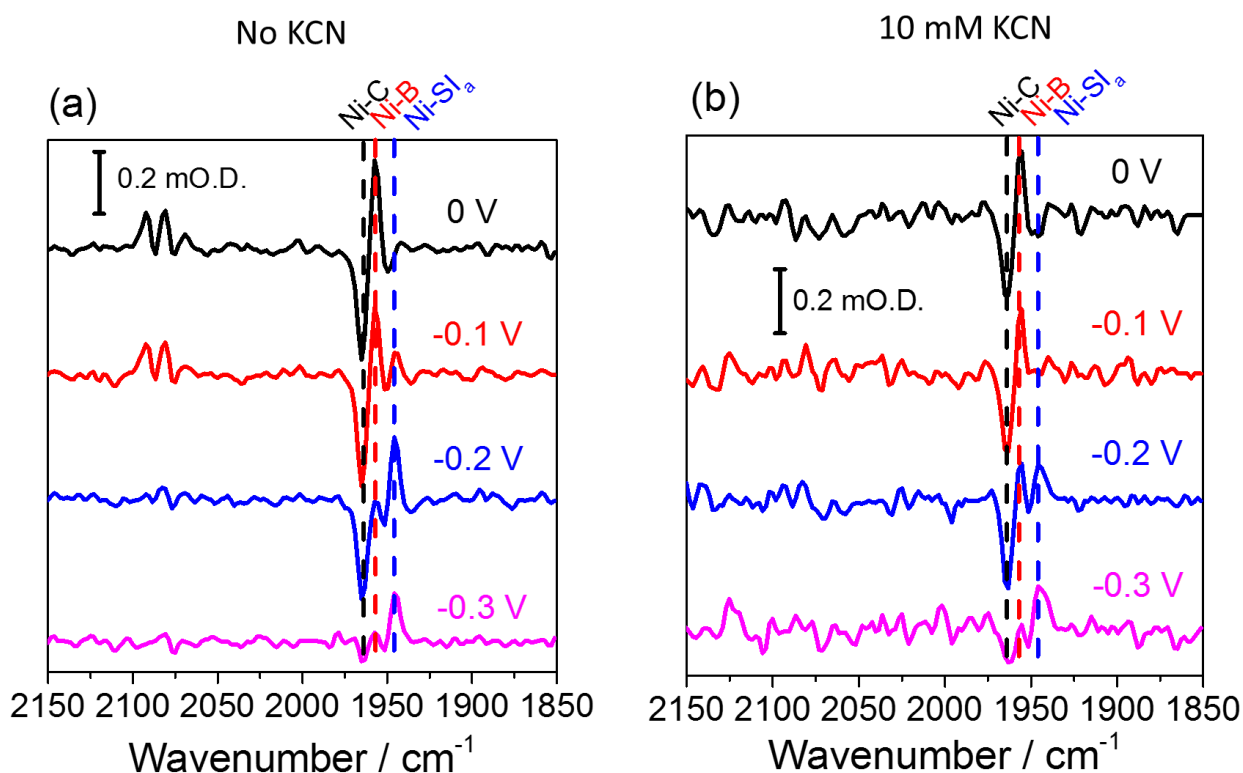


Figure 5-3. ATR-IR redox-triggered difference spectra of Hyd-2 immobilised on surfactant-modified BP2000. Background spectra were collected at -0.4 V, followed by potential steps in 0.1 V intervals. Each spectrum was recorded 20 min after the potential step: (a) without cyanide present; and (b) with 10 mM KCN present. All spectra were recorded in N₂-saturated buffer (pH 7).

5.2.3 Discussion and summary

Results of cyclic voltammetry from Suzannah Hexter show that inactivation of Hyd-1 and Hyd-2 by cyanide is a potential-dependent process (Figure 5-1). The introduction of cyanide accelerates the high potential inactivation, but it does not have any effect on the activity of either enzyme at lower potentials. This feature suggests that cyanide targets the active site of the enzyme, but not reactions with a species directly involved in the catalytic cycle, because such an interaction would result in a decrease in H₂ oxidation regardless of electrode potential, like CO^{73,121} (Chapter 6) and isocyanide (Chapter 7) inhibitions. It is more likely that cyanide reacts with a species which is in rapid equilibrium with active states and can be removed by oxidation/reduction readily as the applied potential is changed.

Chronoamperometric experiments on Hyd-2 indicated that cyanide can establish a new limiting current when it is introduced at high potentials, and the rate of inactivation depends on the concentration of KCN and the substrate, H₂. The rate increases to a limiting value as the cyanide concentration is raised and decreases as [H₂] is increased.⁸³ The observations suggest that cyanide sets up a new equilibrium in Hyd-2 between active and inactive forms. Further studies were carried out by spectroscopy to see if the cyanide-inactivated species can be observed. However, the only species detected in the presence of cyanide for Hyd-2 is Ni-B (with the formation rate being largely enhanced) by either EPR in a freeze-quench method with 1 s time resolution,⁸³ solution ATR-IR spectroelectrochemistry⁸³ or PFIRE (*this Thesis*, even for the experiments

carried out with 1 s time resolution, *data not shown*). The observation is in sharp contrast to inhibitions imposed by its isoelectronic π -acid ligands, CO^{73,121} (Chapter 6) and isocyanides (Chapter 7). Those inhibitors are found to react with the Ni-SI_a state to form the inhibited species, which can be detected by FTIR spectroscopy. However, the spectroscopic results of Hyd-2 is fairly consistent with the argument made earlier revealed from cyclic voltammetry by PFE: the inhibitory effect of cyanide only occurs in a narrow potential window, which suggests that cyanide reacts with a species, which is not directly involved in the catalytic cycle, but is in rapid equilibrium with active states (*vide supra*). Therefore, the possible reasons why the inhibited species cannot be detected by spectroscopy are as follows: (i) the intermediate species just exist transiently, below the time resolution that we were able to achieve; (ii) concentration of the intermediate at any time is not high enough to be detected.

Figure 5-4 depicts a possible pathway of how cyanide promotes Ni-B formation. Well-characterised states (Ni-SI_a, Ni-SI_r and Ni-B) are shown in blue. Red arrows indicate a pathway leading to the formation of the Ni-B state under normal conditions (in the absence of cyanide), whereas green arrows indicate a possible pathway for Ni-B formation which may occur in the presence of cyanide. A rate law equation derived from this proposed mechanism gives good fits to the currents from chronoamperometry for determination of inactivation rate.⁸³ Although, a detailed mechanism of Ni-B formation under normal conditions still remains unclear, in electrochemical terms, it is a “CE” process.¹ Possibly, the key chemical step of forming Ni-B is to get a H₂O molecule in

place in the active site, which converts Ni-SI_a to Ni-SI_r, as depicted in Figure 5-4, and leads to coordination of a OH⁻ ligand to the Ni. Then the following rapid electrochemical step, i.e. the oxidation of Ni(II) to Ni(III), can trap the hydroxide ligand at the bridging position, forming Ni-B eventually. This process is slow under anaerobic conditions.⁴⁸ So what is the role of CN⁻ in promoting the formation of Ni-B? CN⁻ is a better nucleophile than OH⁻ and H₂O. When CN⁻ is present, it coordinates to Ni(II) in a terminal fashion quickly, serving as a kinetically preferred product (compared to a terminal Ni(II)-OH species), and facilitates the oxidation of Ni(II) to Ni(III). However, the binding of CN⁻ to Hyd-2 is fairly weak, supported by a fitted $K_d = 0.84$ mM,⁸³ and a number of examples about labile bonding of Ni(III)-cyanido complexes.^{122,123} And thus, a better bridging ligand, OH⁻, subsequently replaces CN⁻ to stabilise Ni(III) in the active site, completing the formation of Ni-B, which is the thermodynamic product.

In sum, in the presence of cyanide, the formation of Ni-B is largely promoted. The cyanide inhibition is easily reversed because it is simply the reductive activation of Ni-B.

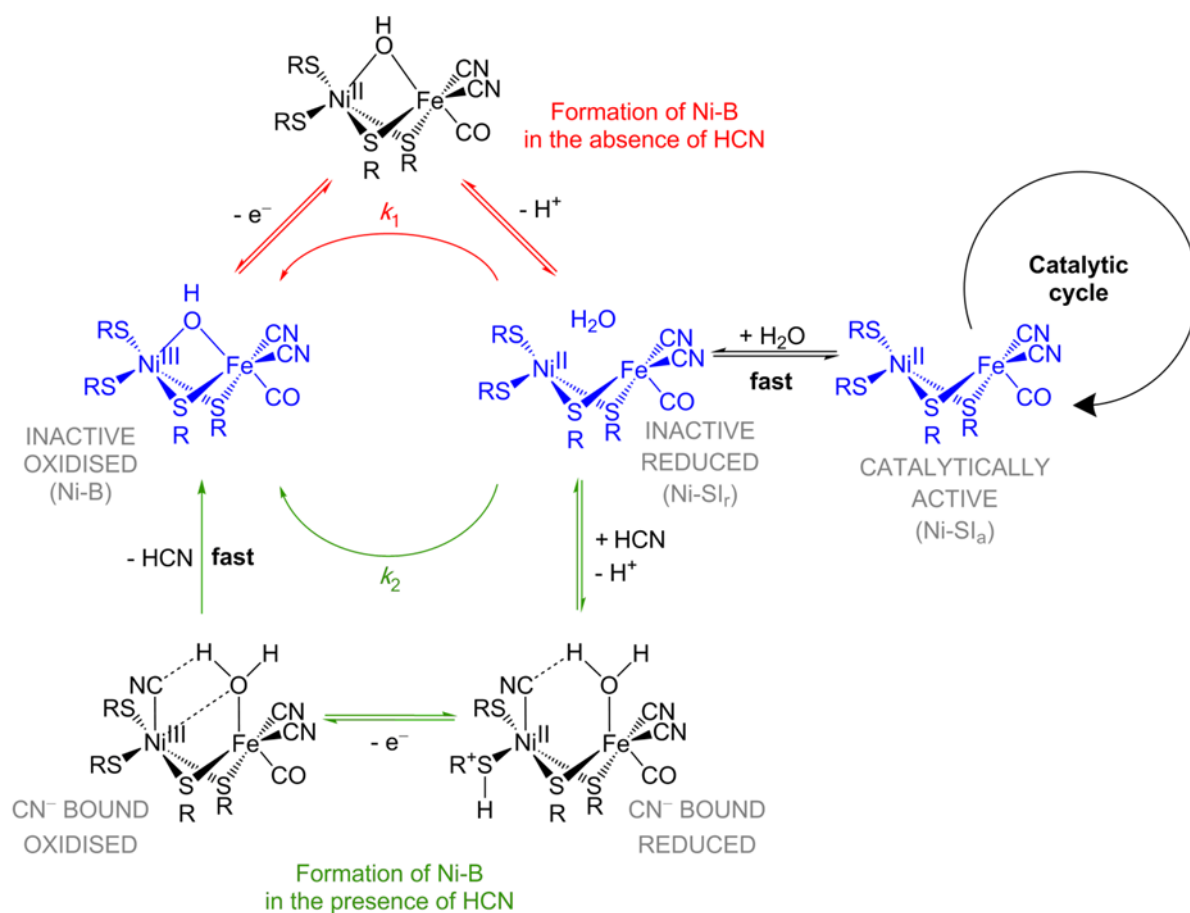


Figure 5-4. A proposed mechanism of how cyanide facilitates the formation of Ni-B. Well-characterised states are shown in blue. Red arrows indicate a pathway leading to the formation of the Ni-B state under normal conditions (in the absence of cyanide), whereas green arrows indicate a possible route for Ni-B formation which may occur in the presence of cyanide. Figures are adopted and reprinted with permission from ref ⁸³. Copyright © 2014, American Chemical Society

5.3 Hyd-1

Results of cyclic voltammetry by PFE from Suzannah Hexter show that the inhibitory effect of cyanide on H₂ oxidation is not specific to Hyd-2, as a similar behaviour is observed for Hyd-1 at potentials more positive than -100 mV at pH 7 (see Figure 5-1).⁸³ However, no further spectroscopic evidence was obtained for the reaction of Hyd-1 with cyanide, so PFIRE is performed herein for a detailed study.

5.3.1 Slightly more Ni-B forms in the presence of cyanide under N₂

Figure 5-5 depicts a set of difference ATR-IR spectra of Hyd-1 under N₂ (pH 7) in different concentrations of cyanide; the spectrum collected at -0.6 V without cyanide present was used as reference. The potential was poised at -0.09 V throughout the whole experiment during changes of cyanide concentrations in the cell, and each spectrum was collected 25 min after introducing cyanide to give final concentrations of 0, 2 and 5 mM in the cell in contact with the enzyme particle film. When cyanide is absent, stepping the potential to -0.09 V results in loss of the Ni-L and Ni-R states (characterised by ν_{CO} of 1877, 1914 and 1922 cm⁻¹ in the negative direction), and formation of the Ni-SI_a and Ni-B states (revealed by positive bands of ν_{CO} at 1929 and 1943 cm⁻¹) as depicted in pink, panel a. After introducing 2 mM cyanide into the spectroelectrochemical cell (shown in blue, panel b), Ni-B increases slightly by approximately 0.1 mO.D., associated with losing an equal amount of Ni-SI_a (measured by the peak height of ν_{CO}). Finally, when 5 mM cyanide is introduced at the same potential, the

amount of Ni-B does not change, but a new band at 1899 cm^{-1} appears (panel c), which is formed from the conversion of Ni-SI_a (see the difference spectrum triggered by the change of cyanide concentration from 2 mM to 5 mM, panel d). The new species is denoted as Ni-X in this Thesis, as it has not been characterised and reported previously.

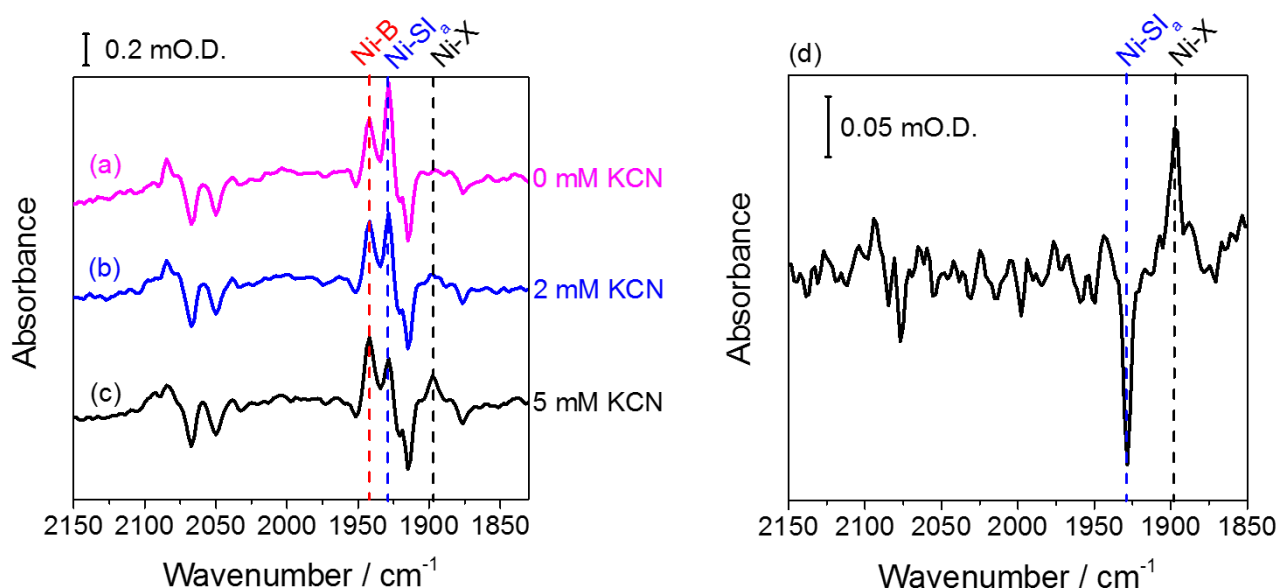


Figure 5-5. ATR-IR difference spectra of Hyd-1 immobilised on BP2000 at -0.09 V with different concentrations of cyanide: (a) 0 mM (—); (b) 2 mM (—); and (c) 5 mM KCN (—). The background spectrum was collected at -0.6 V without cyanide present. Panel (d): A difference spectrum of Hyd-1 triggered by the change of cyanide concentration from 2 mM to 5 mM; the potential was poised at -0.09 V. All spectra were recorded in N₂-saturated buffer (pH 7).

5.3.2 An irreversible species (Ni-X) forms at high concentrations of KCN

A detailed potential titration experiment of Hyd-1 in intervals from -0.6 V up to +0.376 V was performed to examine when the new species of Ni-X starts to appear. Larger potential steps were used in potential regions where there is little change in absorbance of active site ligands, whereas small potential steps were employed in potential regions where changes are more pronounced. An aliquot of KCN stock solution was injected at -0.6 V, giving a final concentration in the cell of 5 mM. The experiment was conducted in N₂-saturated buffer (pH 7). Figure 5-6 shows the results presented as difference spectra, in which a spectrum collected at -0.6 V was used as reference for subtraction. Ni-X can be seen to appear at -0.178 V, and keeps growing during the rest of oxidative titration. It is coexistent with Ni-B at the end of experiment at +0.376 V. The appearance of Ni-X is slightly later than the Ni-SI_a state, which starts forming at -0.258 V. The formation of Ni-X is irreversible: it did not disappear when the potential was stepped back down to -0.6 V, or bubbling H₂ into the cyanide solution to reduce the enzyme and to make cyanide escape from the solution in a gaseous form as HCN, or flowing rinsing buffer solution into the cell at low potentials. An analogous titration experiment was also carried out with 2 mM KCN present, but the peak at 1899 cm⁻¹ was not seen.

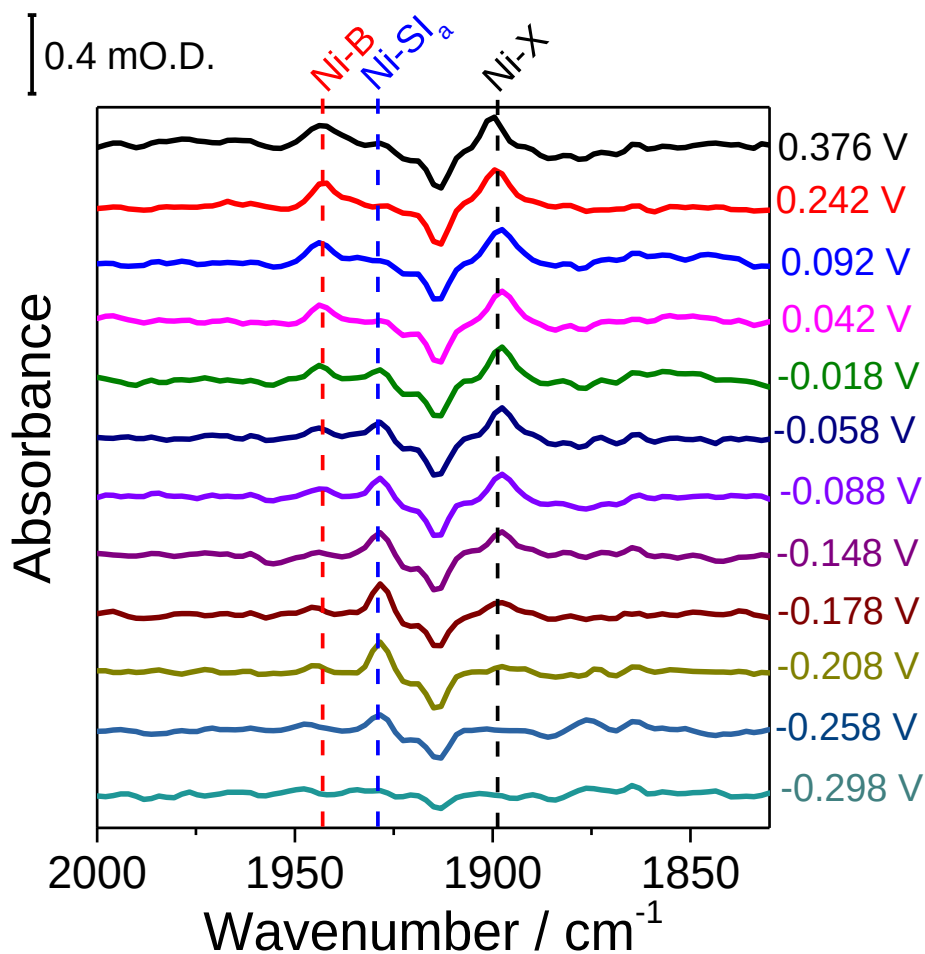


Figure 5-6. ATR-IR redox-triggered difference spectra of potential titration experiment of Hyd-1, in which an aliquot of KCN stock solution was injected at -0.6 V to give a final concentration of 5 mM in the cell (pH 7). A spectrum collected at -0.6 V was used as reference for subtraction. Only the region attributed to absorptions of endogenous CO ligand is shown. The new species with an absorption band at 1899 cm^{-1} is denoted as Ni-X, as it has not been characterised and reported previously.

5.3.3 The origin of vibrational band at 1899 cm⁻¹

In order to clarify whether the absorption band at 1899 cm⁻¹ of Hyd-1 in the presence of CN⁻ is contributed by endogenous CO ligand or exogenous cyanide ligand coordinating to the active site, a set of experiment was carried out on an isotope-labelled Hyd-1 sample, which has an endogenous ¹³CO ligand in the active site, and on a normal wild-type enzyme (Figure 5-7, presented as difference spectra). The experiments were carried out as follows: a potential at -0.358 V was applied to the enzyme samples first, for collecting background spectra, followed by stepping the potential up to +0.242 V. Panel (b) shows the redox-triggered difference spectrum for the isotope-labelled Hyd-1 sample in the presence of 30 mM KCN (pH 6). The spectrum clearly shows that the entire set of CO bands shifts to lower wavenumbers compared to the spectrum of a normal wild-type Hyd-1 (panel a); two negative bands at 1870, 1877 cm⁻¹, and two positive bands at 1898 and 1856 cm⁻¹ are seen. The peak at 1856 cm⁻¹ was not present when the experiment was carried out in the absence of cyanide (panel c) for the isotope-labelled Hyd-1 sample. Vibrational bands at 1870, 1877 and 1898 cm⁻¹ are in good agreement with the predictions calculated by equation [5-1] for isotope shift, using values of $\nu(^{12}\text{CO})$ of Ni-R_{III} (1914 cm⁻¹), Ni-R_{II} (1922 cm⁻¹), and Ni-B (1943 cm⁻¹). The peak at 1856 cm⁻¹ also agrees well with the isotope shift from 1899 cm⁻¹ calculated by equation [5-1], indicating that this vibrational band comes from the intrinsic CO ligand in the active site.

$$\frac{\nu(^{13}\text{CO})}{\nu(^{12}\text{CO})} = \sqrt{\frac{\mu(^{12}\text{CO})}{\mu(^{13}\text{CO})}} = 0.977 \dots\dots\dots [5-1]$$

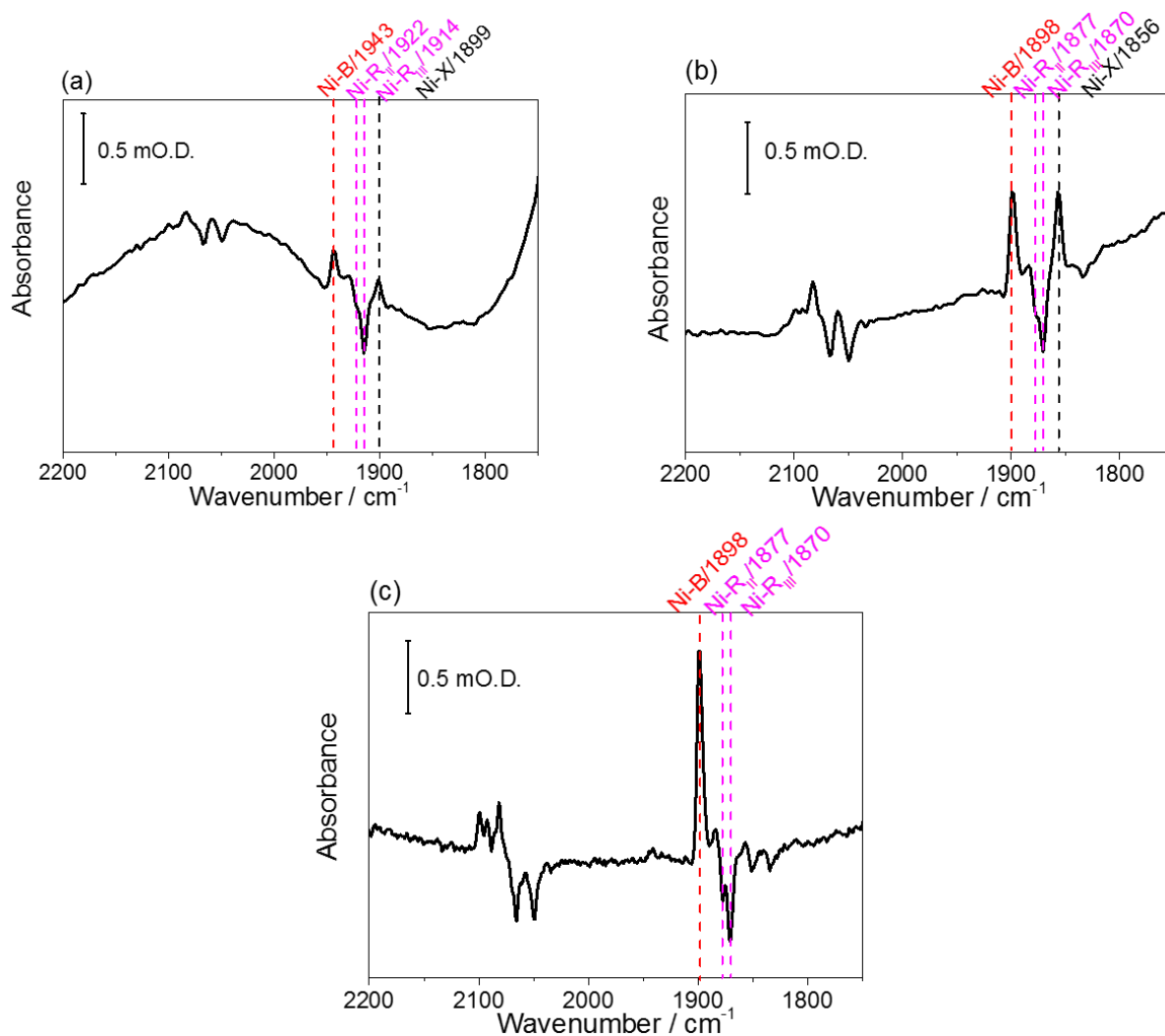


Figure 5-7. ATR-IR redox-triggered difference spectra (+0.242 V minus -0.358 V) of: (a) a normal wild type Hyd-1 in 30 mM KCN (pH 6); and (b), (c) a ^{13}C O-labelled Hyd-1 with and without 30 mM KCN present (pH 6). Only the CO bands were shifted to lower wavenumbers upon ^{13}C isotope labelling.

5.3.4 Discussion and summary

The spectroscopic evidence shows that cyanide has the same effect on Hyd-1 as Hyd-2: the equilibrium in redox states is changed upon introducing cyanide to contact the enzyme sample; Ni-B increases slightly promoted by cyanide (2 mM) under N_2 (see Figure 5-5). However, the phenomenon is not as pronounced as Hyd-2, since the formation of Ni-B in Hyd-1 is intrinsically faster, as revealed from PFE (Figure 5-1, panel b). The cyclic voltammetry was carried out at a scan rate of 4 mV s^{-1} under 1 bar H_2 . When cyanide is absent (the red traces), significant amount of Ni-B forms already at high potentials for Hyd-1, even under 1 bar H_2 (the Ni-B formation rate is found to be in inverse proportion to the concentration of H_2)⁸³, indicated by the feature of reactivation from the oxidative inactive state on the reverse scan when potential is swept to values lower than +0.1 V. In contrast, this feature is not seen for Hyd-2 under comparable conditions (Figure 5-1, panel a). The reactivation from Ni-B for Hyd-2 can be seen in cyclic voltammetry at a slower scan rate of 1 mV s^{-1} , which provides time for the slow process of Ni-B formation proceeding (see Figure 2-4).

At high concentrations of cyanide, i.e. greater than or equal to 5 mM, an irreversible species (Ni-X) forms for Hyd-1, with the characteristic band at 1899 cm^{-1} . The irreversible species is not observed for Hyd-2 when a higher concentration of cyanide solution, 10 mM, was used (*cf.* Figure 5-3). Detailed ATR-IR spectroscopic study on an isotope-labelled Hyd-1 sample (which has an endogenous ^{13}CO ligand in the active site) confirms the band at 1899 cm^{-1} is from the endogenous CO ligand (see Figure 5-7). The formation of Ni-X is from

the conversion of Ni-SI_a (Figure 5-5, panel d), implying that cyanide interacts with Ni-SI_a to have a permanent coordination to the active site, resulting in a downward shift of vibrational energy by 30 cm⁻¹ of the endogenous ν_{CO} .

In sum, cyanide also promotes Ni-B formation for Hyd-1, albeit the effect is not as pronounced as Hyd-2 under N₂ revealed by PFIRE, due to the inherent differences between these two enzymes. A high concentration of cyanide solution leads to a permanent coordination of cyanide to the active site of Hyd-1, and this phenomenon is reminiscent of the irreversible cyanide inhibition of [NiFe]-hydrogenase from *A. vinelandii* at its oxidised state, revealed by spectrophotometric assay.⁸² These features suggest that subtle variants exist among [NiFe]-hydrogenases, even if they are from the same organism.

Chapter 6: CO Inhibition of [NiFe]-Hydrogenases

The aim of this Chapter is to provide a quick review of the well-characterised inhibitor, CO, towards [NiFe]-hydrogenases. Although, *E. coli* Hyd-1 and Hyd-2 follow general responses to CO compared with other hydrogenases from different organisms revealed by PFE,^{1,8,67} no further spectroscopic evidence has been obtained for the reaction between Hyd-1/Hyd-2 and CO. Therefore, PFIRE is performed herein for a detailed study. The results presented in this Chapter are used for further comparison with the inhibitory behaviour imposed by isocyanides in the next Chapter.

6.1. Introduction

6.1.1 CO inhibition of [NiFe]-hydrogenase from *DvMF*

CO serves as a severe inhibitor towards hydrogen-cycling catalysts,¹²⁴ among which hydrogenases also cannot be spared. However, unlike Pt-based catalysts, activities of [NiFe]-hydrogenases are easily restored upon removal of CO, as revealed by PFE.¹ The CO inhibition of [NiFe]-hydrogenases has been well characterised; CO shows negligible potential-dependent inhibition, i.e. it inhibits the whole potential window where H₂ cycling occurs.¹

In 2002, crystallographic studies from Ogata *et al.* on hydrogenase from *DvMF* provided direct structural-evidence that the exogenous CO binds to the Ni, which also implies that this is the initial site for dihydrogen activation.⁷⁴ However, the coordination of CO is not in a linear fashion (Figure 6-1); the angle of the bound CO distributes between 161° and 136° among different crystals.

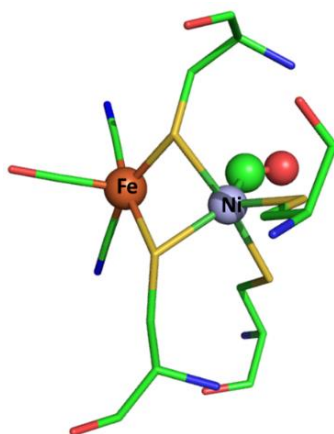


Figure 6-1. Crystal structure of [NiFe]-hydrogenase from *DvMF*, revealing how the exogenous CO binds to the active site. (PDB code: 1UBH).⁷⁴ The bimetallic centre and the exogenous CO are shown as spheres; all atoms are represented in standard colours.

Further spectroscopic studies on the same enzyme by Pandelia *et al.* in 2010 showed that CO reacts with the active site at both the Ni-SI_a and Ni-L levels, which have an open bridge to let CO get in for coordination, hindering the catalytic activity.¹²¹ Figure 6-2 depicts the FTIR spectra of Ni-SI_a and the CO-inhibited species, termed Ni-SCO, for *DvMF* [NiFe]-hydrogenase. At -228 mV, Ni-SI_a is the dominant species, with ν_{CO} at 1943 cm⁻¹ associated with asymmetric- and symmetric- stretches of the two cyanide ligands at 2086 and 2074 cm⁻¹. A small proportion of Ni-SI_r is also present at this potential, with a set of characteristic absorptions at 1921 (ν_{CO}), 2070 (ν_{CN}) and 2061 (ν_{CN}) cm⁻¹. Upon introduction of CO at this potential, a strong absorption band at 1941 cm⁻¹ appears, which is attributed to the vibration of the endogenous CO

ligand; vibrational stretches at 2084 and 2071 cm^{-1} are contributed by the asymmetric- and symmetric- stretches of the two cyanide ligands associated with this state. The peak at 2056 cm^{-1} comes from exogenous CO, which was confirmed by ^{13}CO isotope labelling. The spectroscopic feature reveals that the exogenous CO serves a π -acceptor in coordination with the active site; a back donation of electrons from Ni^{II} to its antibonding orbitals decreases the strength of CO bond, leading to a downward shift by 87 cm^{-1} relative to the free CO in the gas phase (2143 cm^{-1}).⁹⁷ Also, FTIR spectroscopy showed that the spectrum of Ni-SCO strongly resembles the one of Ni-SI_a, with only a downward shift by 2-3 cm^{-1} for absorptions of the diatomic ligands in the active site. This revealed that there are only small conformational changes in the active site upon attack by external CO, and there is a slight increase in electron density within the active site imposed by an extra ligand.

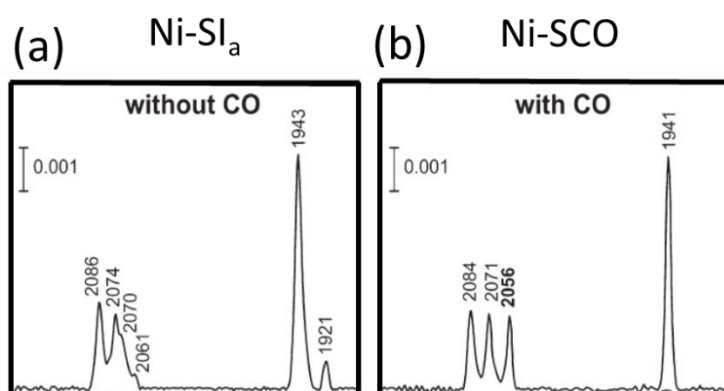


Figure 6-2. FTIR spectra of DvMF [NiFe]-hydrogenase with potential poised at -228 mV without CO present (a) and in the presence of CO (b), pH 7.4. Figures are adapted and reprinted with permission from ref ¹²¹. Copyright © 2010, Elsevier.

6.1.2 PFE studies on interactions of Hyd-1 and Hyd-2 with CO

Studies of Hyd-1 and Hyd-2 inhibition by CO have been carried out using PFE by Lukey *et al.* (Figure 6-3).⁶⁷ The results indicated that CO acts as a reversible inhibitor towards both enzymes. The inhibition constant (K_I) values for H₂ oxidation were determined to be $51 \pm 6 \mu\text{M}$ for Hyd-1 at -0.06 V and $3 \pm 1 \mu\text{M}$ for Hyd-2 at -0.175 V, and the apparent K_I value for proton reduction for Hyd-2 was calculated as $11 \pm 3 \mu\text{M}$ at -0.56 V. The results are in line with observations from other [NiFe]-hydrogenases,^{1,8} i.e. the standard hydrogenases (Hyd-2 here) not only show far greater sensitivity to O₂, but also are more sensitive to inhibition by CO during H₂ oxidation than O₂-tolerant MBHs (Hyd-1 here).

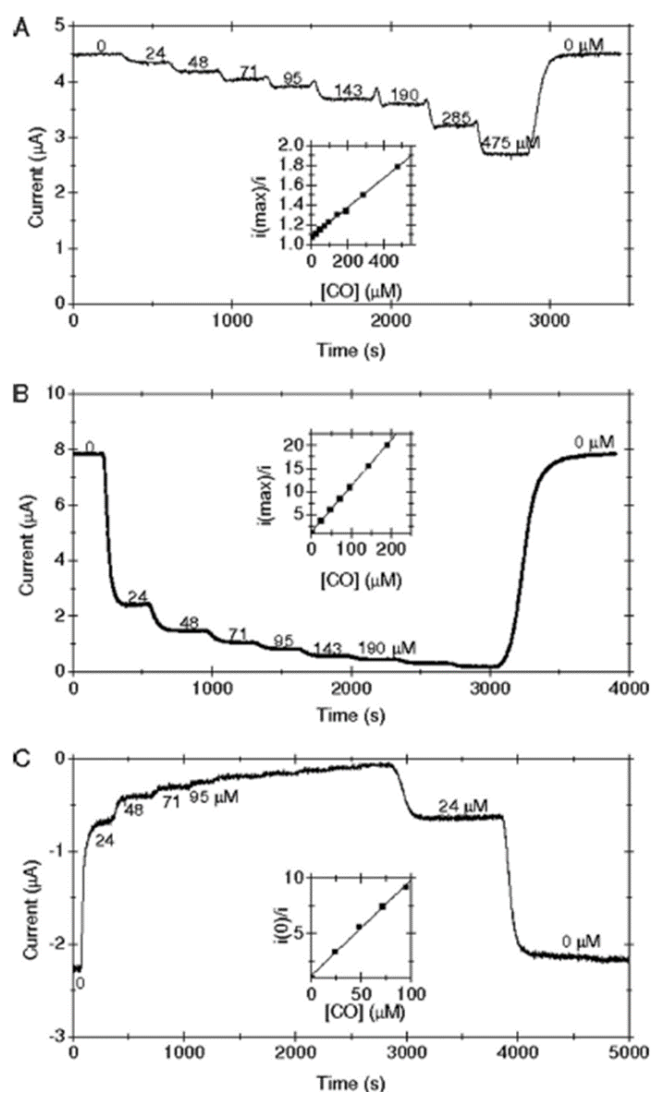


Figure 6-3. Chronoamperometry traces of Hyd-1 (panel A) and Hyd-2 (Panel B and C) for inhibition constant determinations at -0.06, -0.175, and -0.56 V for Panel A, B and C, respectively. The experiments were carried out under 20% H_2 in argon (panel A and B)/argon (panel C) with increasing partial pressures of CO (the resulting concentration of CO in the electrochemical cell solution is indicated). Other conditions: pH 6, 20 °C. Figures are adapted and reprinted with permission from ref ⁶⁷, Copyright © 2010, by the American Society for Biochemistry and Molecular Biology

6.2. Results and Discussion

6.2.1 Hyd-2

Figure 6-4, panel (a) depicts an absolute spectrum of Hyd-2 recorded under Ar at -100 mV (pH 6). At this potential, Ni-SI_a is the dominant species, as revealed by the characteristic absorptions at 1946 (ν_{CO}), 2086 (ν_{CN}) and 2075 (ν_{CN}) cm^{-1} (*cf.* Table 4-2). A spectrum collected at the same potential but with 50% CO in argon present is depicted in panel (b), in which an intense absorption at 1944 cm^{-1} associated with three weaker vibrational bands at 2055, 2073 and 2083 cm^{-1} are seen. These vibrational bands show a close similarity to the reported infrared absorptions of Ni-SCO of *DvMF* [NiFe]-hydrogenase at 1941, 2056, 2071 and 2084 cm^{-1} , with the absorption of exogenous CO at 2056 cm^{-1} .¹²¹ Thus, for Hyd-2, assigning the vibrational band at 2055 cm^{-1} as exogenous CO stretch is sensible, because these two enzymes were found to share a similar electronic environment within the active site. The appearance of this exogenous CO peak can be confirmed with confidence by examining a difference spectrum (panel c), which is calculated from the spectrum collected under 50% CO minus the one recorded under Ar at -100 mV (“b” minus “a”). In which a band at 2055 cm^{-1} is clearly visible in the positive direction. Loss of the Ni-SI_a state is not well resolved in the difference spectrum (indicated by the characteristic ν_{CO} at 1946 cm^{-1} shown in the negative direction), as the spectroscopic feature of Ni-SCO strongly resembles the one of Ni-SI_a. Also, the absorptions of the entire set of endogenous diatomic ligands are shifted to lower wavenumbers by 2-3

cm^{-1} in Ni-SCO compared with those of Ni-SI_a, and this is attributed to the slight increase in electron density within the active site upon being attacked by the exogenous CO, sharing the same trend with *DvMF* [NiFe]-hydrogenase.

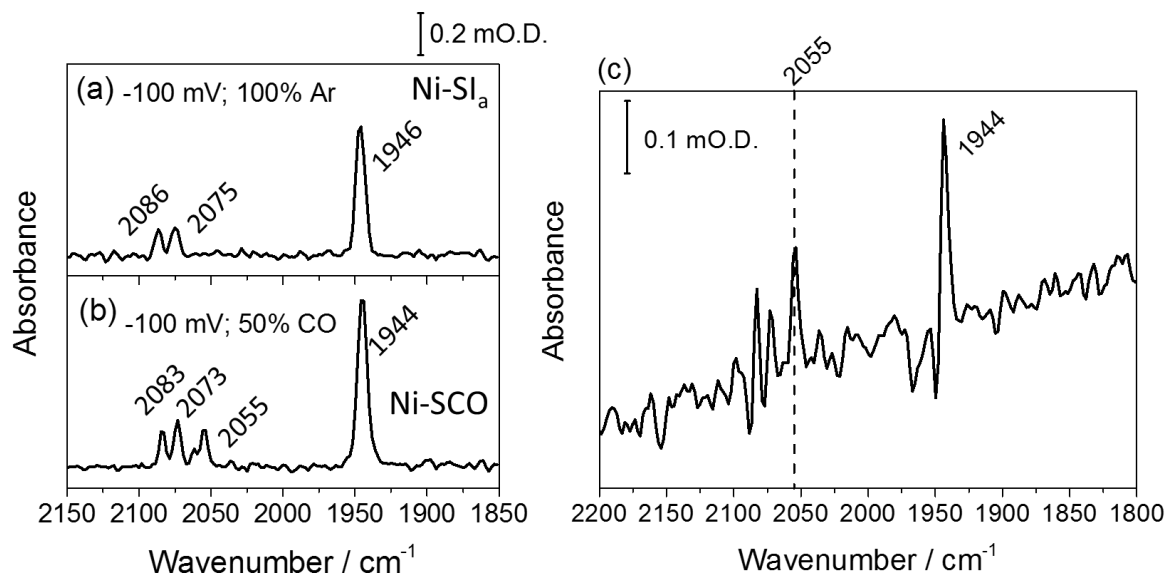


Figure 6-4. Absolute ATR-IR spectra of Hyd-2 collected at -100 mV under 100% Ar (panel a), and under 50% CO in argon (panel b). Panel (c): a difference spectrum of Hyd-2 calculated by “(b) minus (a)”. Other conditions: pH 6; 25 °C.

Figure 6-5 depicts cyclic voltammograms of Hyd-2 recorded in the ATR-IR cell (pH 7). The current trace collected under 50% H₂ in argon is shown in black, (i). Changing gas composition to 50% H₂ and 50% CO leads to loss of catalytic activities in both H₂ oxidation and proton reduction (red trace, ii), indicating that the inhibition occurs over the whole potential window for H₂ cycling (-0.5 V to +0.3 V). The catalytic activities can be restored readily upon removal of CO; a switch of gas composition back to 50% H₂ in argon results in regaining

both activities (blue trace, iii). Underachievement of full recovery in oxidation current is attributed to some degree of film loss during experimental operation.

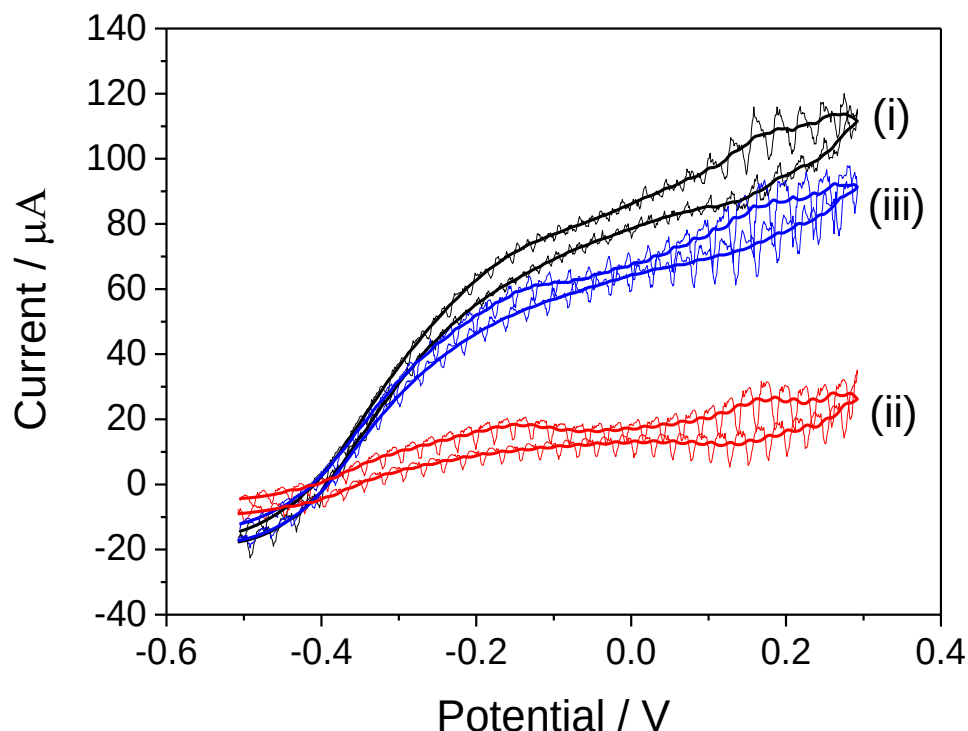


Figure 6-5. Cyclic voltammograms of Hyd-2 collected in the ATR-IR cell under 50% H₂ in argon (black trace, i); and 50% H₂ with 50% CO (red trace, ii). A voltammogram collected after removing CO by a switch of gas composition back to 50% H₂ in argon (blue trace, iii) shows that the CO inhibition of Hyd-2 is reversible (it should be noted that the failure to fully recover all the H₂ oxidation current might be due to some degree of film loss during experimental operation). Other conditions: pH 7; scan rate, 10 mV s⁻¹; room temperature; buffer solution flow rate, 46.2 mL min⁻¹.

Figure 6-6 shows chronoamperometry traces at selected electrode potentials under 50% H₂ in argon (panel a, black), and 50% H₂ with 50% CO (panel a, red) at pH 7, on a single sample of immobilised Hyd-2 on surfactant-modified

particles. It is clear to see that the loss of activities occurs over a wide potential range for both H_2 oxidation and proton reduction when 50% CO is present, in which the inhibitory effect is slightly more pronounced at more positive potentials, i.e. approximately 82% and 78% of activity in H_2 oxidation is inhibited by CO at +0.042 V and -0.358 V, whereas the reduction current decreases by *ca.* 73% at -0.558 V. Difference spectra calculated from absolute spectra collected under catalytic turnover at +0.042 and -0.558 V (“with CO” minus “without CO”) are shown in (b) and (c) respectively. In which, loss of Ni-C and Ni-R, and appearance of Ni-SCO is clearly visible at both potentials. Loss of the Ni-SI_a state cannot be well resolved in the difference spectrum due to the close similarity in the spectroscopic feature between Ni-SI_a and Ni-SCO. The observation of loss of Ni-C and Ni-R can be rationalised well by considering the interference imposed by CO to the dynamic equilibrium in the catalytic cycle (*cf.* section 4.1.1). For H_2 oxidation, when CO (a competitive inhibitor to H_2) is present, it reacts with the Ni-SI_a state and blocks the site where H_2 can get activated, and thus the concentrations of Ni-R and Ni-C decrease significantly, as the formation of these states follows the cleavage of H_2 upon the substrate attacking Ni-SI_a. As for proton reduction, although Ni-R is the initial state reacting with proton for H_2 production (proposed on the basis of microscopic reversibility, see discussion in section 4.3.1), regeneration of this state is via the route of Ni-SI_a and Ni-C by sequential one-electron reduction. Thus, the attack of CO on Ni-SI_a would also destroy the dynamic equilibrium among the active states causing loss of Ni-C and Ni-R as well.

Moreover, the phenomenon that the inhibitory effect of CO is more pronounced at more positive potentials during catalysis can also be understood well by referring to the state distributions under catalytic turnover (*cf.* Figure 4-3); concentrations of the state reacting with CO, i.e. Ni-SI_a, are slightly higher at more positive potentials applied. Thus, stronger interference from CO would be imposed to the catalytic cycle. Also, the recovery of the CO-inhibited state by reduction to Ni-R will be slow at higher potentials.

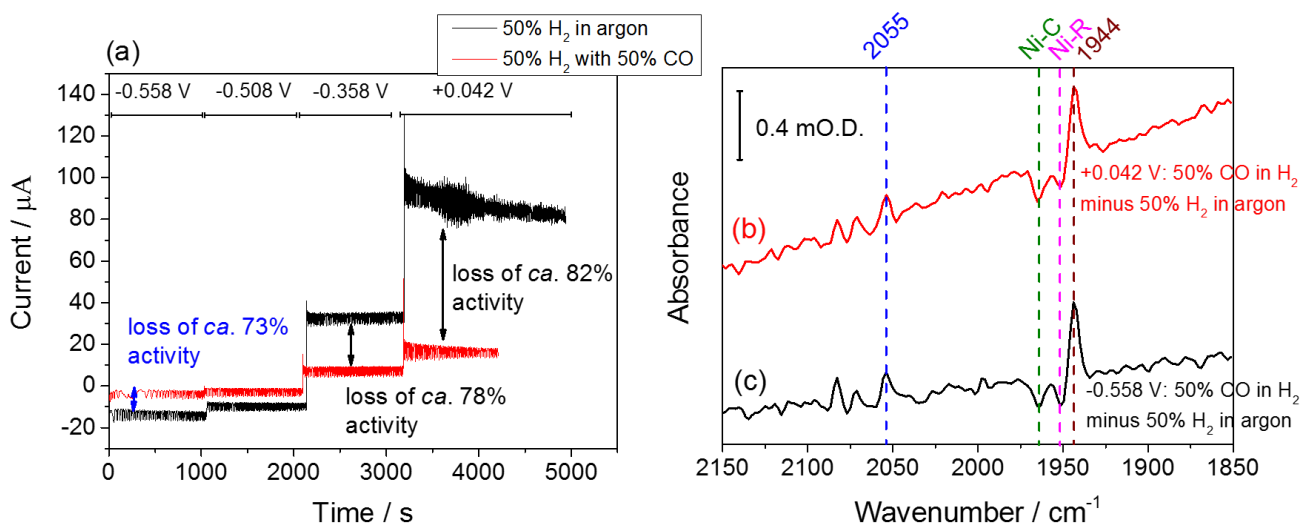


Figure 6-6. (a) Current-time traces of Hyd-2 collected in the ATR-IR cell under 50% H₂ in argon (black) and 50% H₂ with 50% CO (red), pH 7; (b),(c) difference spectra calculated by absolute spectra collected under catalytic turnover at +0.042 and -0.558 V; “with CO” minus “without CO”.

6.2.2 Hyd-1

(data are provided by Ricardo Hidalgo and Eleanor Hall)¹²⁵

Figure 6-7 depicts the cyclic voltammograms of Hyd-1 collected in the ATR-IR cell under 50% H₂ in N₂ (black) and 50% H₂ with 50% CO (red) at pH 6 on a single sample of immobilised Hyd-1. It is clear to see that a significant catalytic H₂ oxidation current is still observed in the presence of 50% CO for Hyd-1, i.e. *ca.* 66.6% of activity is retained at +0.042 V. Whereas, only 18% of activity is retained for Hyd-2 at the same potential under 50% CO (*cf.* Figure 6-6). This investigation is envisaged for Hyd-1, as it is an O₂-tolerant MBH; it is well known that this type of hydrogenase is more resistant to CO inhibition when the enzyme is turning over under H₂.^{8,33,67}

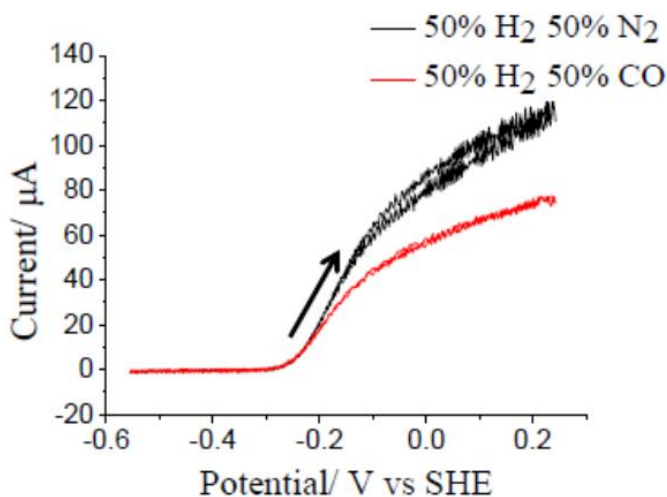


Figure 6-7. Cyclic voltammograms of Hyd-1 collected in the ATR-IR cell under 50% H₂ in nitrogen (black trace); and 50% H₂ with 50% CO (red trace). Other conditions: pH 6; scan rate, 0.5 mV s⁻¹; room temperature; buffer solution flow rate, 42.3 mL min⁻¹. This figure was provided by Eleanor Hall.¹²⁵

Chapter 6. CO inhibition of [NiFe]-hydrogenases

The interaction between CO and the active site of Hyd-1 is further ascertained by PFIRE, depicted in Figure 6-8 as a difference spectrum. A spectrum collected at -115 mV under 100% N₂ was used as reference, at which the Ni-SI_a state is found to be the dominant species, accompanied by a small proportion of Ni-L. When the gas composition was changed to 50% CO in nitrogen at the same potential, a set of positive peaks at 2081, 2071, 2060, and 1928 cm⁻¹ was observed in the difference spectrum (“with CO” minus “without CO”). This set of peaks shows close similarity to the Ni-SCO state of *A. vinosum* hydrogenase (2082, 2069, 2060, 1929).^{117,118} *A. vinosum* hydrogenase was found to share a similar electronic environment with Hyd-1 based on FTIR spectroscopy (*cf.* Table 4-1), and therefore features of this enzyme in infrared absorptions of the diatomic ligands serve as a good reference point for assistance in identifying species for Hyd-1. Thus, by the comparison of these two sets of data, the peak at 2060 cm⁻¹ for Hyd-1 is assigned to the absorption of exogenous CO coordinating to the active site. Also, there is a weak negative peak at 1877 cm⁻¹ (ν_{CO}), corresponding to loss of the Ni-L_{II} species (*cf.* Table 4-1). Loss of the Ni-SI_a state cannot be well resolved in the difference spectrum due to the close similarity in the spectroscopic feature between Ni-SI_a and Ni-SCO. These investigations provide strong evidence that the CO inhibitor also attacks the active site of Hyd-1 at the Ni-SI_a level, which is consistent with observations from other [NiFe]-hydrogenases.^{8,121}

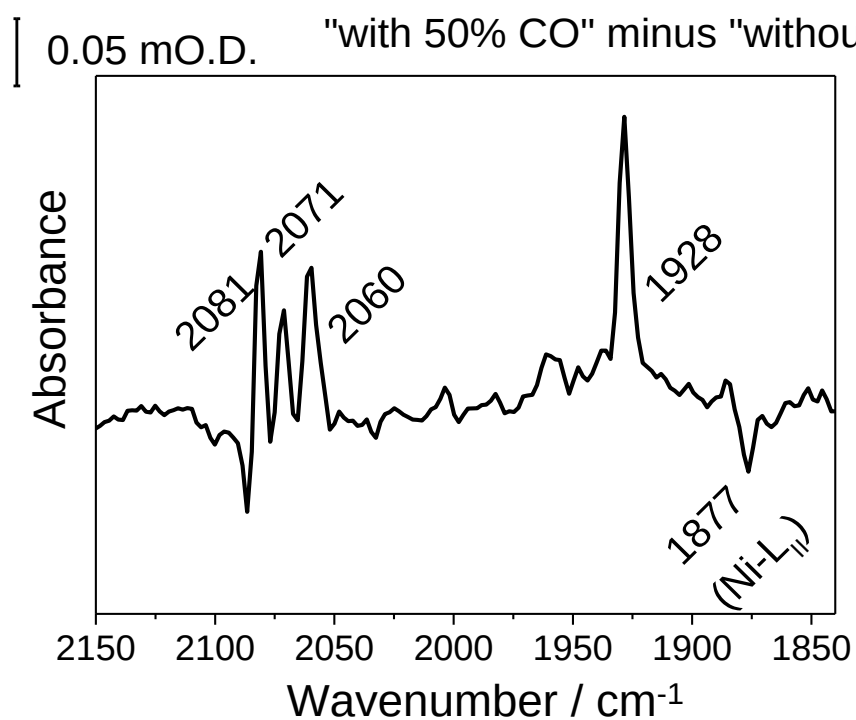


Figure 6-8. A difference spectrum of Hyd-1 at -115 mV triggered by changing gas composition from 100% N₂ to 50% CO in N₂; the spectrum collected under 100% N₂ was used as reference. The experiment was carried out by Eleanor Hall.

6.3. Summary

The results presented in this Chapter by PFIRE have shown that the responses of Hyd-1 and Hyd-2 to CO are similar to other [NiFe]-hydrogenases, i.e. CO interacts with Ni-SI_a, and the spectra of Ni-SCO of Hyd-1 and Hyd-2 also display close similarity to those from a range of microorganisms.⁸ Moreover, the phenomenon of slightly stronger inhibition of CO at more positive potentials applied for Hyd-2 under catalytic turnover is also in good agreement with state distributions in response to catalysis: at more positive potentials, the concentration of Ni-SI_a is higher (*cf.* Figure 4-3), and thus, the inhibitory effect is more pronounced due to the stronger interference that could be imposed by CO on the dynamic equilibrium. Also, for Hyd-1, the fact that it is more resistant to CO under catalytic turnover can also be rationalised well by the same argument, regarding the state distributions governed by unusually high redox potentials of the FeS clusters: Ni-R is always the dominant species during H₂ oxidation for Hyd-1 (*cf.* Figure 4-6, and discussion in section 4.3.2), and thus only a relatively small amount of Ni-SI_a is available to be attacked by CO compared with that for Hyd-2, meaning that the interference imposed by CO to the dynamic equilibrium between activate states is smaller.

Chapter 7: Reversible Reactions of [NiFe]-Hydrogenases with Isocyanides

In this chapter, isocyanides (R-NC, R= methyl, ethyl and *n*-butyl) are reported for the first time as reversible inhibitors of *E. coli* Hyd-1, suggested by PFE and PFIRE. The tuneable R-group of isocyanide makes it serve as a good probe into the steric constraints of the catalytic pocket of the enzyme. The findings reveal that there is spacious room within the active site.

7.1. Introduction

In Chapter 5, studies of cyanide inhibition of Hyd-1 and Hyd-2 show that cyanide serves as a promoter to facilitate Ni-B formation. However, since the binding between cyanide and the enzyme molecule is fairly weak, together with the subsequent reactions occurring rapidly, the bound cyanide species serving as an intermediate for Ni-B facilitation are not detected. An exception occurs in the case of Hyd-1; an irreversible species forms at high cyanide concentration (pending clarification). This observation motivates us to exploit isocyanides (RNC) as a probe for further understanding coordination chemistry of the active site in [NiFe]-hydrogenases.

Isocyanides possess a divalent carbon, which is not common among stable organic compounds. They serve as strong carbon ligands with high reactivity towards transition metals, contributed by a pair of nonbonding electrons in the sp-hybridised orbital on the terminal carbon. Isocyanides are isoelectronic with π -acid ligands of CO and CN^- , but have much larger dipole moment, i.e. 34- fold larger than that of CO.⁸⁵ They have also been reported as inhibitors of several metalloproteins, such as nitrogenase,⁸⁴ carbon monoxide dehydrogenase (CODH),⁸⁵ and haem proteins.⁸⁶ However, isocyanide inhibition of [NiFe]-hydrogenases was not found in our literature survey. The only example addressing inhibitory effect of isocyanides on hydrogenases is studies of [Fe]-hydrogenase by Shima and co-workers.^{126,127} However, [Fe]-hydrogenases are

phylogenetically distinct from [NiFe]-hydrogenases, and have a completely different architecture (*cf.* Figure 1-3).

FTIR of Isocyanides

Figure 7-1 depicts FTIR spectra of the alkyl isocyanides used in spectroelectrochemical studies in this Chapter. The FTIR spectrum of neat methyl isocyanide (panel a, black) shows an intense absorption at 2166 cm^{-1} , which is attributed to the $\text{N}\equiv\text{C}$ stretch. In contact with buffer solution (pH 6, mixed buffer), the band shifts to 2187 cm^{-1} (panel a, red, 84 mM) contributed by the hydrogen bonding interaction between water molecules and the carbon of isocyanides, which has been suggested to be the site of proton acceptor in hydrogen bonding.¹²⁸ The spectrum of neat ethyl isocyanide is depicted in panel b, black. Upon the substitution group being changed to longer ethyl chain, which has a stronger electron-donating ability relative to methyl group, the ν_{NC} shifts to lower wavenumber at 2149 cm^{-1} . The aqueous ethyl isocyanide (300 mM, panel b, red) shows three characteristic bands at 2168, 2185, and 2149 cm^{-1} . The former two split peaks are contributed by Fermi resonance, which occurs when a vibrational mode with a strong absorbance, and the resonance of this mode with other vibrations leads to enhancement in their intensities. In the case of ethyl isocyanide here, the $\text{N}\equiv\text{C}$ stretch resonates with the modes within the ethyl group, giving rise to two strong absorption bands at 2168 and 2185 cm^{-1} for the aqueous isocyano stretch.¹²⁹ The band at 2149 cm^{-1} arises from the isocyano group in the organic microenvironment of the ethyl isocyanide micelle-like droplets, due to the poor solubility of ethyl isocyanide. Panel c

depicts the spectra of *n*-butyl isocyanide. An intense absorption of the neat form at 2147 cm^{-1} is seen (black). Two peaks are investigated for aqueous *n*-butyl isocyanide (panel c, 380 mM, red). Because the solubility is poorer as the alkyl chain is longer, an intense band at 2147 cm^{-1} coming from the isocyano group trapped in the micelle droplets is also investigated (with the phenomenon being more pronounced); the weak shoulder at 2171 cm^{-1} is attributed to the absorption of aqueous form.

It should be noted that the phenomenon of Fermi resonance for peak splitting in aqueous isocyanide solution is unique to the ethyl group among alkyl isocyanides, and the reason is not clear yet.¹²⁹

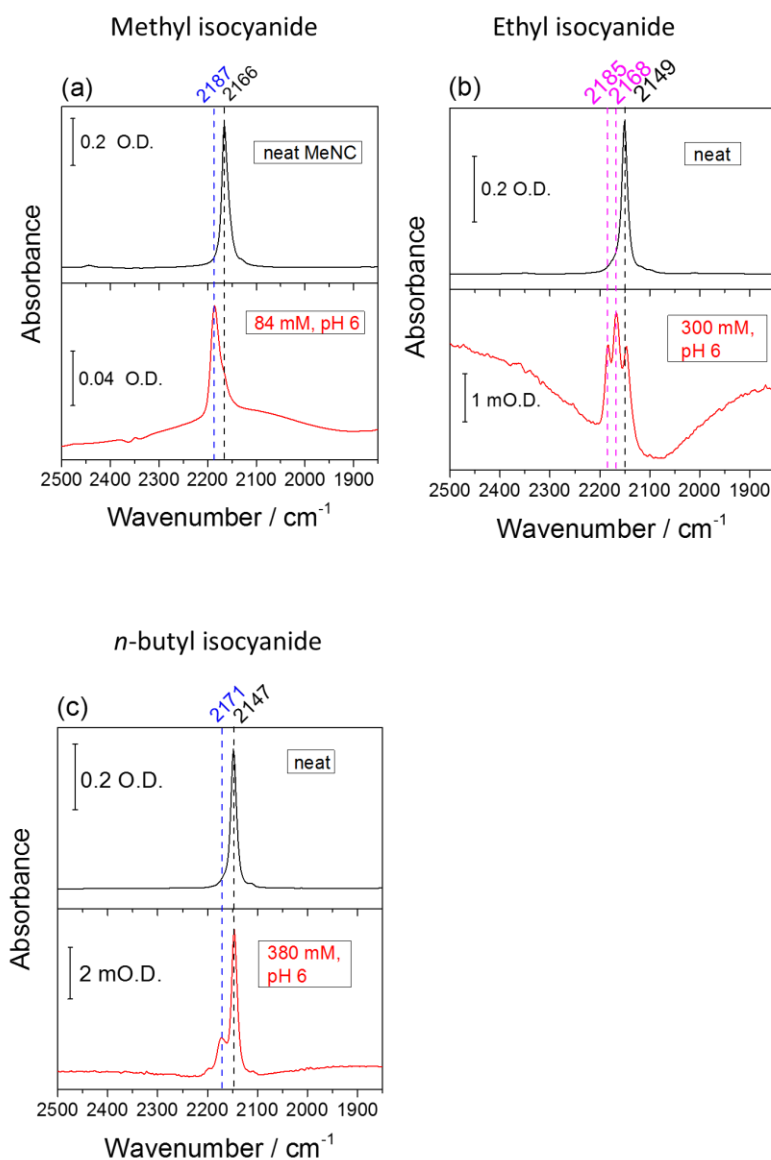


Figure 7-1. FTIR spectra of alkyl isocyanides: methyl isocyanide (panel a); ethyl isocyanide (panel b); and *n*-butyl isocyanide (panel c) used in spectroelectrochemical experiments in this Chapter. The neat form is shown in black, and the spectra collected in the presence of buffer solution (pH 6, mixed buffer) is represented in red.

7.2 Methyl isocyanide (MeNC): a reversible inhibitor of Hyd-1 and Hyd-2

Results

(This project is in collaboration with a former Part II student, Benjamin Aucott.¹³⁰ MeNC was synthesised following a method reported by Schuster *et al.*,¹⁰² and was prepared by Pathinan Paengnakorn.)

Protein film electrochemistry of Hyd-1 (on a RDE) with MeNC

Chronoamperometric experiments were conducted to study how MeNC affects Hyd-1 on a RDE, as depicted in Figure 7-2. Panel (a) shows that H₂ oxidation of Hyd-1 (revealed by a positive current) is rapidly inhibited by MeNC. The electrode was poised at -100 mV, and at time = 330, 620, 860 and 1110 s (indicated by the arrows), aliquots of MeNC were injected into the electrochemical cell to give final concentrations of 4.56, 9.15, 13.8 and 18.4 mM, respectively. Immediate decreases in catalytic currents upon successive injections are seen. Panel (b) reveals that the H₂ oxidation activity of Hyd-1 is easily restored at -100 mV by diluting MeNC in the electrochemical cell. The experiment was started with almost complete loss of activity of Hyd-1 under 10% H₂, which was achieved by the use of 1.68 M MeNC to inhibit the enzyme. A slight recovery of catalytic activity was seen after diluting the MeNC concentration in the cell each time, which was carried out by removing 1 mL aliquots of solution from the cell, followed by adding back 1 mL rinsing solution

(pH 6; pre-saturated with 10% H₂). The catalytic current can almost be restored to its original value by continuing the dilution process. Cyclic voltammetry experiments on a RDE were then performed to have an overview of how MeNC affects Hyd-1 over a wider potential range. Figure 7-3 depicts a set of voltammograms recorded on the same film: (i) without MeNC; (ii) with 17.2 mM MeNC present; (iii) after swapping all MeNC solution in the cell with rinsing buffer. The results clearly show that MeNC inhibits the whole potential window, i.e. -0.2 V to +0.34 V, where the catalysis of H₂ oxidation occurs. The catalytic activity can be almost restored in the clean buffer solution; a slight drop in oxidation current in the voltammogram compared with the one before introducing MeNC is attributed to slight film loss during buffer solution exchange.

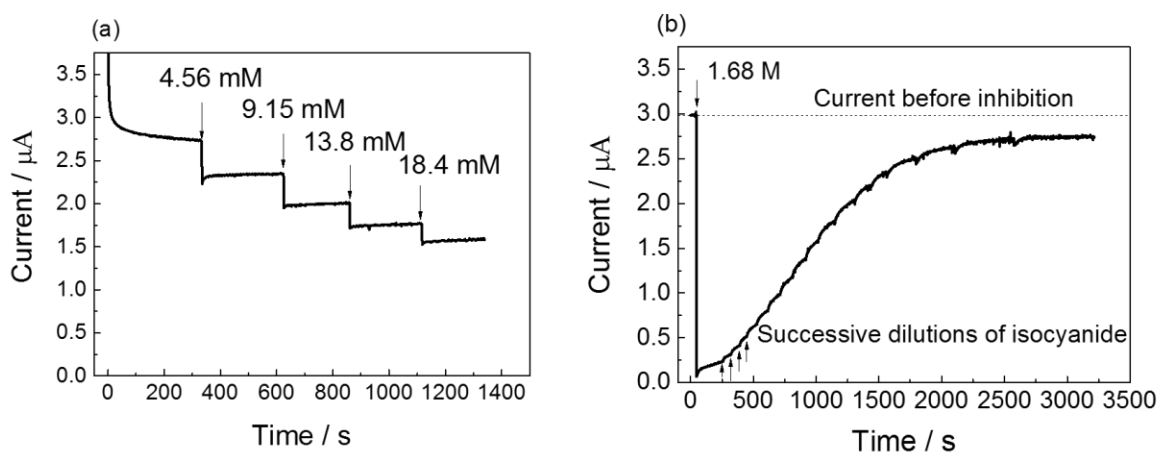


Figure 7-2. Current traces of chronoamperometric experiments (on a RDE), which show how MeNC affects H_2 oxidation of Hyd-1. Panel (a): the potential was poised at -100 mV, and aliquots of MeNC stock solution were injected at time points indicated by the arrows, with the final concentrations in the cell solution shown alongside. Panel (b): dilutions of MeNC in the electrochemical cell at -100 mV. Aliquots of the methyl isocyanide stock solution were added to the cell to give a concentration of 1.68 M first, causing almost complete loss of activity. The MeNC was then diluted by removing 1 mL aliquots of solution from the cell, followed by refilling with 1 mL aliquots of rinsing solution each time. Other conditions: mixed buffer, pH 6.0; 25°C; 10% H_2 ; electrode rotation rate, 2000 rpm.

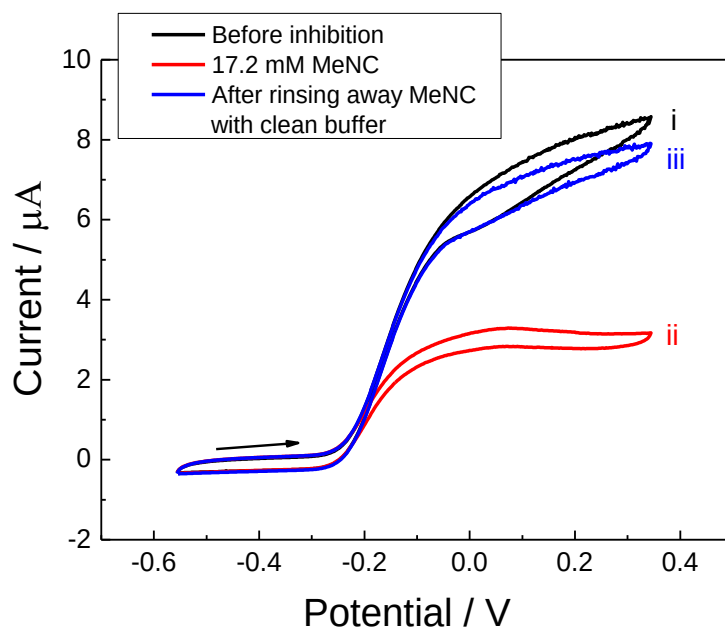


Figure 7-3. Cyclic voltammograms of Hyd-1 (experiments were carried out on the same film on a RDE): (i) without MeNC; (ii) with 17.2 mM MeNC present; and (iii) after removal of MeNC with rinsing buffer (pre-saturated with 10% H_2). The arrow shows the direction of the scan. Other conditions: mixed buffer, pH 6.0; 25°C; 10% H_2 ; electrode rotation rate, 2000 rpm; scan rate, 10 mV s^{-1} .

Protein film electrochemistry of Hyd-2 (on a RDE) with MeNC

Analogous experiments on a RDE were performed to investigate how MeNC inhibits Hyd-2, which has distinct characteristics from Hyd-1, albeit both of them are native enzymes from *E. coli*. Hyd-2 is an O₂-sensitive enzyme and is a good catalyst for H₂ oxidation and production, whereas Hyd-1 shows a very strong bias for H₂ oxidation under physiological conditions. Chronoamperometric results are depicted in Figure 7-4. Panel (a) shows that at -100 mV, MeNC inhibits H₂ oxidation of Hyd-2 quickly. Successive injections of aliquots of MeNC at time = 472, 527, 567, 602, and 632 s to give final concentrations in the cell of 0.55, 1.1, 1.6, 2.2 and 2.7 mM cause immediate drops in the catalytic current. The activity of Hyd-2 is also readily recoverable upon the removal of MeNC, as depicted in panel (b). Swapping 1 mL aliquots of solution from the electrochemical cell with rinsing buffer (pH 6) each time leads to recovery of catalytic current. Cyclic voltammetry experiments were also performed for Hyd-2 to see how potential dependent the inhibition is. Figure 7-5 depicts a set of voltammograms recorded on the same film: (i) without MeNC; (ii) with 0.22 mM MeNC present; (iii) with 2.72 mM MeNC present; and (iv) after swapping all MeNC solution in the cell with rinsing buffer. The results clearly show that MeNC inhibits over the whole potential window, i.e. -0.56 V to +0.34 V, where the catalysis of H₂ oxidation and production occurs. The catalytic activity can be restored almost completely in the clean buffer solution; a slight drop in oxidation current in the

voltammogram compared with the one before introducing MeNC is also attributed to some degree of film loss during buffer solution exchange.

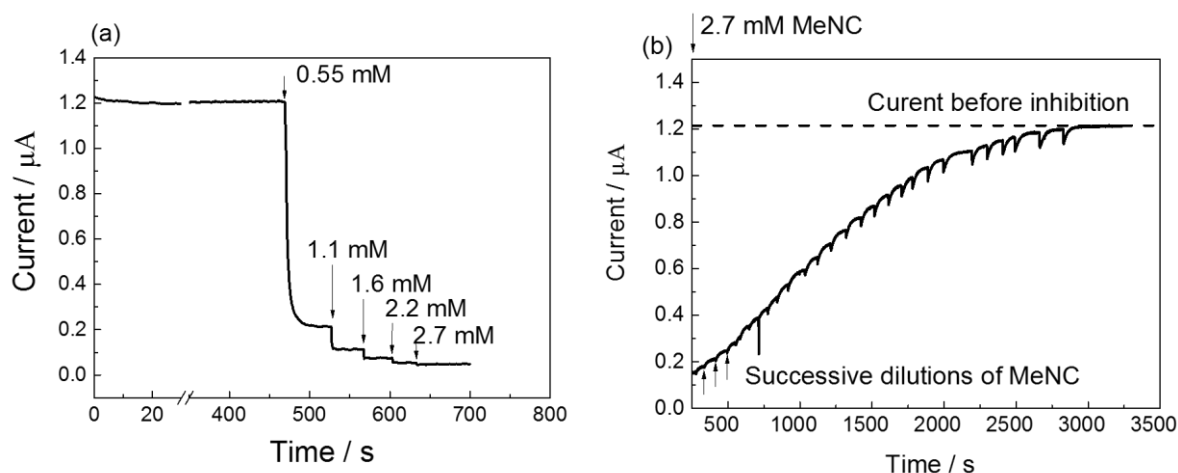


Figure 7-4. Current traces of chronoamperometric experiments (on a RDE), which show how MeNC affects H_2 oxidation of Hyd-2. Panel (a): the potential was poised at -100 mV, and aliquots of MeNC were injected at time points indicated by the arrows, with the final concentrations in the cell solution shown alongside. Panel (b): dilutions of MeNC in the electrochemical cell at -100 mV, with a starting concentration of 2.7 mM. The MeNC was diluted by removing 1 mL aliquots of solution from the cell, followed by refilling with 1 mL rinsing buffer each time. Other conditions: mixed buffer, pH 6; 25 °C; 10% H_2 ; electrode rotation rate, 2000 rpm.

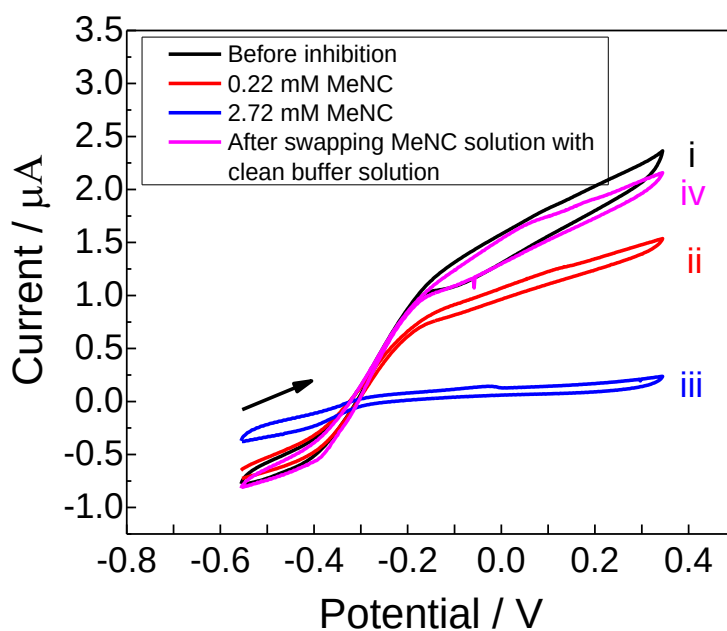


Figure 7-5. Cyclic voltammograms of Hyd-2 (experiments were carried out on the same film on a RDE): (i) without MeNC; (ii) with 0.22 mM MeNC present; (iii) with 2.72 mM MeNC present; and (iv) after removal of MeNC with rinsing buffer. The arrow shows the direction of the scan. Other conditions: mixed buffer, pH 6; 25 °C; 10% H₂; electrode rotation rate, 2000 rpm; scan rate, 10 mV s⁻¹.

Determinations of inhibition constant for Hyd-1 and Hyd-2 with MeNC

Chronoamperometric experiments on a RDE at -100 mV were carried out under different concentrations of substrate (H_2) and inhibitor (MeNC) for inhibition constant (K_I) determinations. Since the current is proportional to the catalytic rate, v , of the enzyme film in PFE (*cf.* section 2.1.6), K_I can be determined by a plot of the reciprocal of the catalytic current against inhibitor concentration, $[I]$, under several $[\text{H}_2]$, i.e. a Dixon plot. The Cornish-Bowden plots of $[\text{H}_2]/v$ against $[I]$ were used to identify the inhibition type (*cf.* section 2.3.3).

A well-established method in PFE for determinations of K_I was adopted in the measurements,¹³¹ and the procedure was carried out as follows: no MeNC was present at the beginning of each measurement, and the buffer solution was in equilibrium with 100% H_2 to give a ‘control’ current for normalisation, since each enzyme film has a different level of activity. Then, the desired H_2/N_2 gas mixture (10%, 15% and 25% by volume) was flowed through the cell until a stable current was obtained. To confirm that the current was not limited by mass transport, it was determined that no enhanced current occurred as the electrode rotation rate was increased. Prior to the injections of MeNC, the flow inlet and outlet of gas to the cell were closed to prevent MeNC vapour being flushed from the cell, which could reduce the accuracy of the concentrations. Then, a series of injections were made quickly. Figure 7-6 depicts a typical chronoamperometric experiment according to the procedure described above, using a set of injections of MeNC to Hyd-1 under 10% H_2 for demonstration.

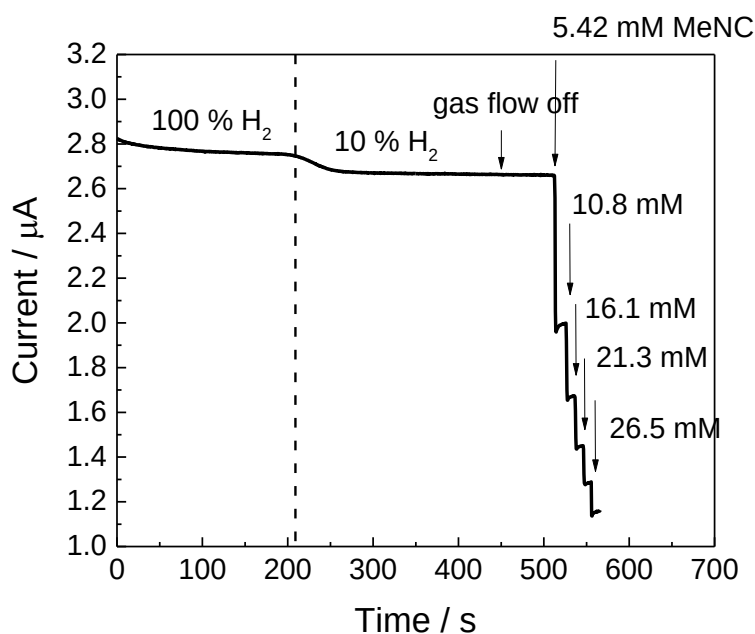


Figure 7-6. A typical chronoamperometric experiment on a RDE for inhibition constant determinations. A set of injections of MeNC under 10% H₂ for Hyd-1 is used for demonstration.

The Cornish-Bowden plot from these experiments on Hyd-1 conducted under 10 %, 15 %, 25 % and 100 % H₂ (Figure 7-7, panel a) shows a set of close to parallel lines, suggesting that the binding of H₂ and MeNC is mutually exclusive for Hyd-1, i.e. a competitive inhibition. The lines intersect in the region of -0.8 to -1.8 mM in the Dixon plot of Hyd-1 (Figure 7-7, panel b), revealing a K_I of between 0.8 and 1.8 mM. Three repeats at 15% H₂ are also shown on the graph. Extrapolating any of these three sets of data shows that the lines intersect in the same region, giving similar ranges for K_I , which indicates that the experiment is reproducible. The middle set (green diamond

symbol) was used for the extrapolation shown in the inset and for the Cornish-Bowden plot.

Analogous experiments were performed for Hyd-2, shown in Figure 7-8. The Cornish-Bowden plot (panel a) depicts a set of close to parallel lines, implying that H₂ and MeNC also bind to Hyd-2 competitively. The Dixon plot (panel b) gives a K_I in the region of 5-20 μ M, which is approximately two orders of magnitude smaller than that of Hyd-1, revealing Hyd-2 is more sensitive to MeNC.

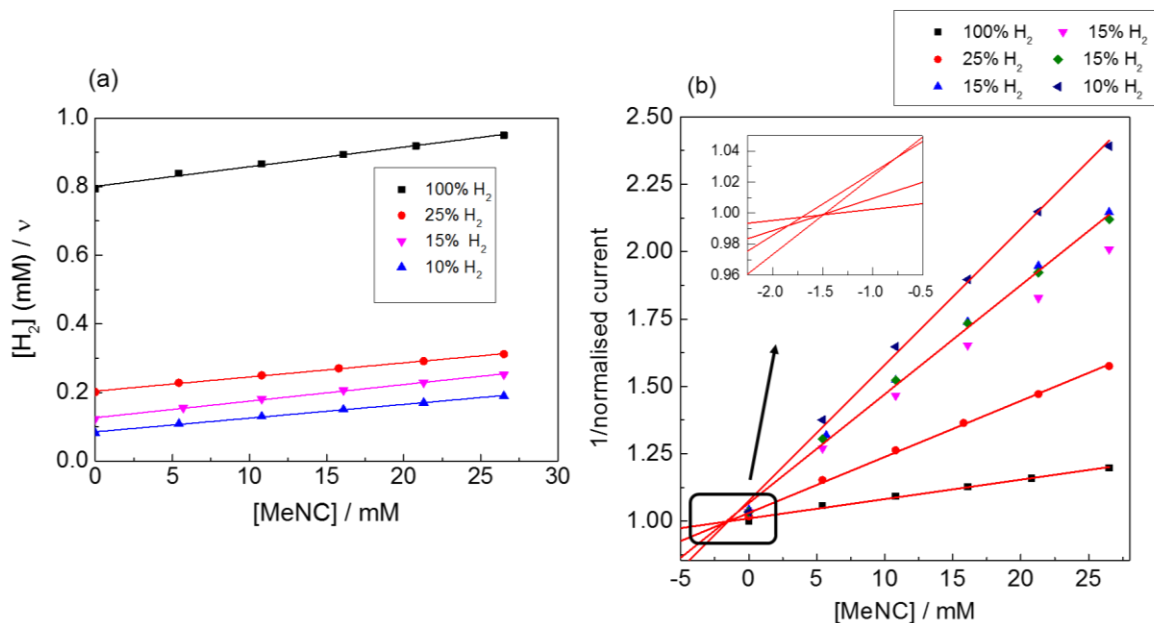


Figure 7-7. Panel (a): a Cornish-Bowden plot for Hyd-1. Panel (b): a Dixon Plot for Hyd-1 with MeNC. The inset shows the region where the lines intersect. Experiments were carried out at 28.5 °C, pH 6.0 (mixed buffer) at -100 mV.

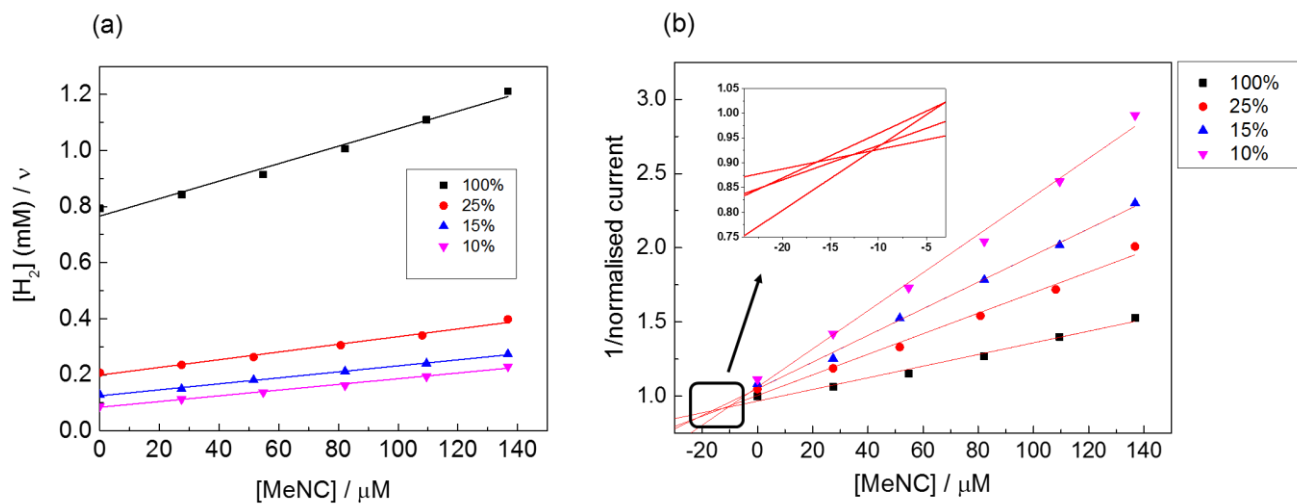


Figure 7-8. Panel (a): a Cornish-Bowden plot for Hyd-2. Panel (b): a Dixon Plot for Hyd-2 with MeNC. The inset shows the region where the lines intersect. Experiments were carried out at 28.5 °C, pH 6.0 (mixed buffer) at -100 mV.

Protein film infrared electrochemistry (PFIRE) of Hyd-1 and Hyd-2 with MeNC

Structural information on how MeNC inhibits Hyd-1 and Hyd-2 can be ascertained by PFIRE under precise potential control. Efficient solution flow for introduction/removal of inhibitors into/from our spectroelectrochemical cell makes it possible to demonstrate the action of MeNC as a reversible enzyme inhibitor *in situ*, which complements evidence from electrochemistry. In this section, all experiments were carried out under non-catalytic conditions for simplification of studies, since a number of states co-exist when the enzyme is turning over under H₂ (*cf.* Chapter 4).

Figure 7-9 depicts PFIRE spectra of Hyd-1. Panel (a) shows an absolute spectrum recorded at -100 mV (pH 6) before introduction of MeNC into the spectroelectrochemical cell. Two vibrational bands of ν_{CO} at 1929 and 1942 cm⁻¹ clearly indicate the presence of Ni-SI_a and Ni-B states at this potential. Panel (b) shows a difference spectrum of Hyd-1, triggered by change of MeNC concentration. Introduction of 8.78 mM MeNC into the cell at -100 mV leads to the appearance of a set of negative peaks at 1929 (ν_{CO}) and 2085 (ν_{CN}) cm⁻¹, consistent with loss of the Ni-SI_a state (*cf.* Table 4-1), and appearance of a new set of positive bands at 1917, 2064, 2073, 2157 and 2187 cm⁻¹, which have not been reported for Hyd-1 before. Most of these peaks are potential dependent, except the one at 2187 cm⁻¹, as shown in redox-triggered difference spectra (Figure 7-10, panel a). The spectrum collected after stepping the potential to -556 mV minus the one recorded at -100 mV shows a set of negative peaks at 2157, 2073 and 1917 cm⁻¹, and a set of positive bands at 2066 (ν_{CN}), 2049 (ν_{CN}) and 1922 (ν_{CO}) cm⁻¹, which is consistent with formation of Ni-R at this potential

(black trace, *cf.* Table 4-1, and ref ⁵¹). A reverse trend is seen when the potential was stepped back to -100 mV again (and minus the one recorded at the former potential of -556 mV), as depicted in green trace. A difference spectrum triggered by flushing out MeNC from the cell (Figure 7-10, panel b) at -100 mV depicts the appearance of a strong positive band at 1929 cm⁻¹ (ν_{CO} , a marker of Ni-SI_a), and a set of negative peaks at 1917, 2064, 2073, 2157 and 2187 cm⁻¹, which reveals that the MeNC inhibition is readily reversible for Hyd-1.

Among the set of new peaks (1917, 2064, 2073, 2157 and 2187 cm⁻¹) formed in the presence of MeNC at -100 mV, the band at 2187 cm⁻¹ is attributed to the aqueous isocyano stretch, ν_{NC} ,¹²⁹ by the fact that it is not potential dependent and appears/disappears upon introduction/removal of MeNC to the cell (*cf.* Figure 7-1, panel a; Figure 7-9, panel b; and Figure 7-10). The peaks at 1917, 2064, and 2073 cm⁻¹, which are in the region of endogenous diatomic ligands of the active site, i.e. 1960-1860 cm⁻¹ for ν_{CO} and 2100-2050 cm⁻¹ for ν_{CN} , are assigned to the characteristic bands of MeNC inhibited species. The peak downshifted by 30 cm⁻¹ relative to the aqueous isocyano group, and which is potential dependent (sharing the same trend with characteristic bands of MeNC inhibited species), is assigned to the bound MeNC (ν_{NC} , 2157 cm⁻¹) to the active site. The observations that Ni-SI_a disappears upon introducing an inhibitor into the cell, and also that the Ni-SI_a state can be regained readily by removal of the inhibitor are reminiscent of CO inhibition of [NiFe]-hydrogenases, in which, exogenous CO burrows into the active site, reacting with the Ni-SI_a state and coordinating to Ni^{II} to form Ni-SCO^{74,121} (see Chapter

6). Thus, in a similar way, the inhibited species formed by reacting with MeNC is denoted as Ni-S_{MeNC} here for further discussion.

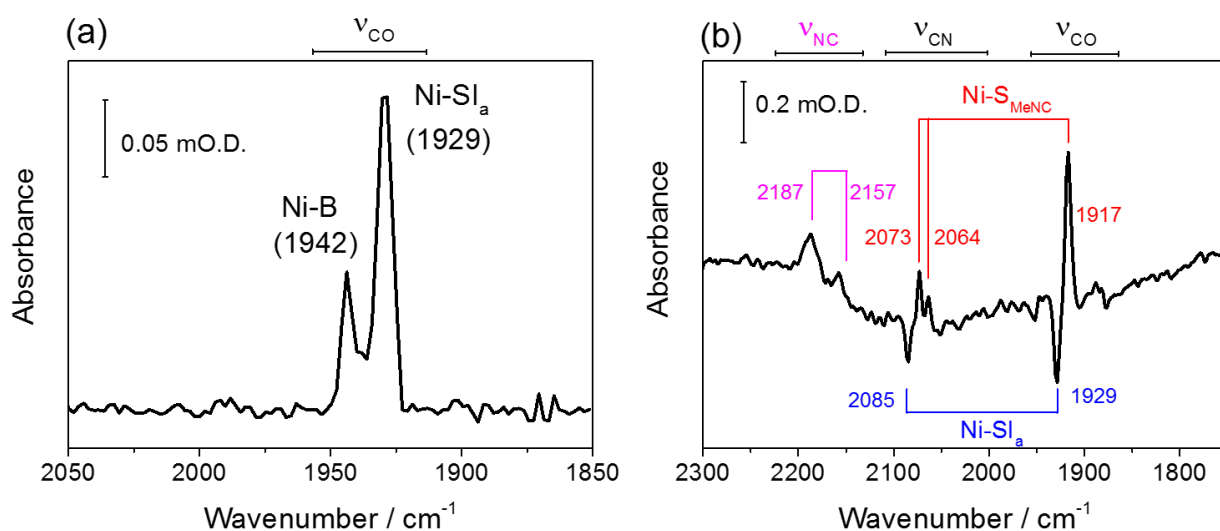


Figure 7-9. ATR-IR spectra of Hyd-1. Panel (a): a baseline-corrected absolute spectrum collected at -100 mV before introduction of MeNC (a clean prism spectrum was used as reference). Only the region of endogenous ν_{CO} is shown. Panel (b): a difference spectrum recorded at the same potential, -100 mV, after introducing 8.78 mM MeNC into the cell; a spectrum collected without MeNC present (shown in panel (a)) was used as reference.

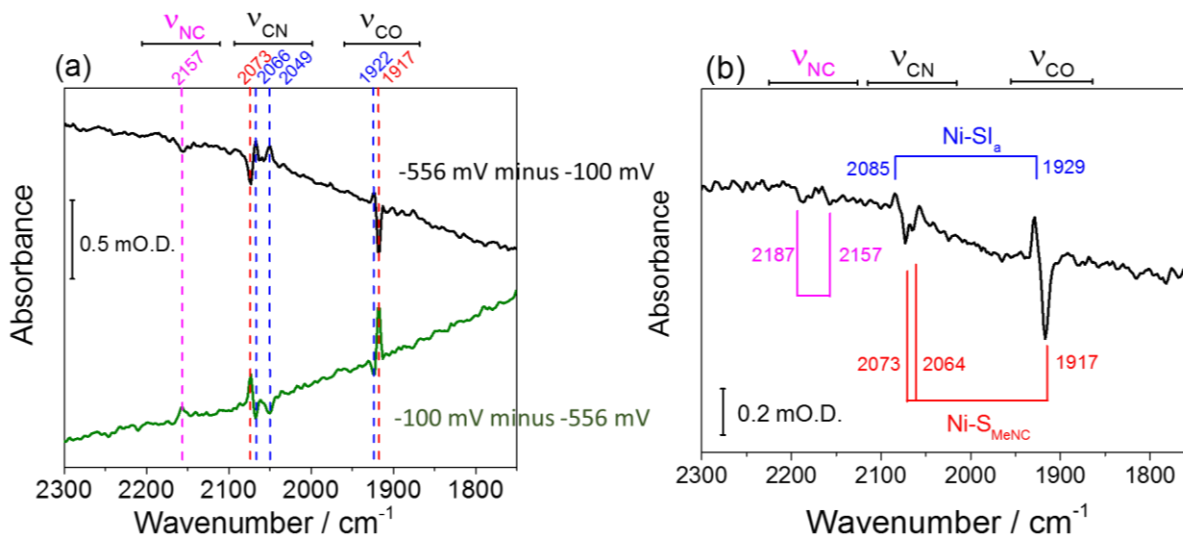


Figure 7-10. Panel (a): redox-triggered difference spectra in the presence of 8.78 mM MeNC. The black trace shows the spectrum collected at -556 mV with the one collected at -100 mV as reference, and the green trace depicts the spectrum recorded after stepping the potential back to -100 mV again, with the spectrum collected at the former potential, -556 mV, as reference. Panel (b): a difference spectrum collected at -100 mV triggered by dilution of MeNC in the cell; a spectrum recorded with 8.78 mM MeNC present was used as reference. Other conditions: pH 6 (mixed buffer); 25 °C.

Similar spectroscopic features are investigated for the MeNC inhibited species for Hyd-2, as depicted in Figure 7-11. Panel (a) shows a difference spectrum at -100 mV (pH 6) triggered by flowing 2.8 mM MeNC into the spectroelectrochemical cell to contact the enzyme film. The introduction of MeNC results in appearance of a sharp negative peak at 1946 cm^{-1} (ν_{CO}), corresponding to loss of Ni-SI_a (*cf.* Table 4-2), and an intense positive band at 1933 cm^{-1} , which is assigned to the intrinsic ν_{CO} of Ni-S_{MeNC} for Hyd-2. A strongly absorbing band at 2217 cm^{-1} (FWHM: *ca.* 48 cm^{-1}) also forms upon introducing MeNC. Because this band is fairly broad compared with the peaks of not only the endogenous diatomic ligands (FWHM: *ca.* 7 cm^{-1}), but also the bound MeNC for Hyd-1 (2157 cm^{-1} ; FWHM: *ca.* 7 cm^{-1}) and aqueous MeNC (2187 cm^{-1} ; FWHM: *ca.* 20 cm^{-1}), this peak was not assigned as coming from the bound MeNC for Hyd-2. Instead, it is more likely that this broad band is contributed by trapped aqueous MeNC inside either the protein cavities or the self-assembled micelle of DDAB on the carbon surface. Trapped ligands inside the protein cavities is a common feature in studies of ligand migration upon photolysis for myoglobin, such as CO and NO migrations,^{132,133} in which the band of trapped CO was found at similar wavenumbers with that of free ligand (either slightly higher or lower by *ca.* 7 cm^{-1}). As for Hyd-2 here, although the possible trapped MeNC with absorption band at 2217 cm^{-1} is higher than the free ligand by 30 cm^{-1} , this assignment is still sensible because MeNC is more polar than CO, and thus the influence on bond strength by the surrounding environment would be more pronounced. Instead, the tiny peak (with the

intensity is just above the noise) at 2161 cm^{-1} is suspected to be the bound MeNC for Hyd-2, which is similar to the wavenumber investigated for Hyd-1, i.e. at 2157 cm^{-1} .

The inhibition of MeNC is also readily reversible for Hyd-2 (Figure 7-11, panel b). Rinsing the cell with clean buffer solution (pH 6) leads to a conversion from Ni-S_{MeNC} to Ni-SI_a, supported by characteristic bands at 1933 cm^{-1} (ν_{CO} of Ni-S_{MeNC}) in the negative direction, and a positive peak at 1946 cm^{-1} (ν_{CO} of Ni-SI_a).

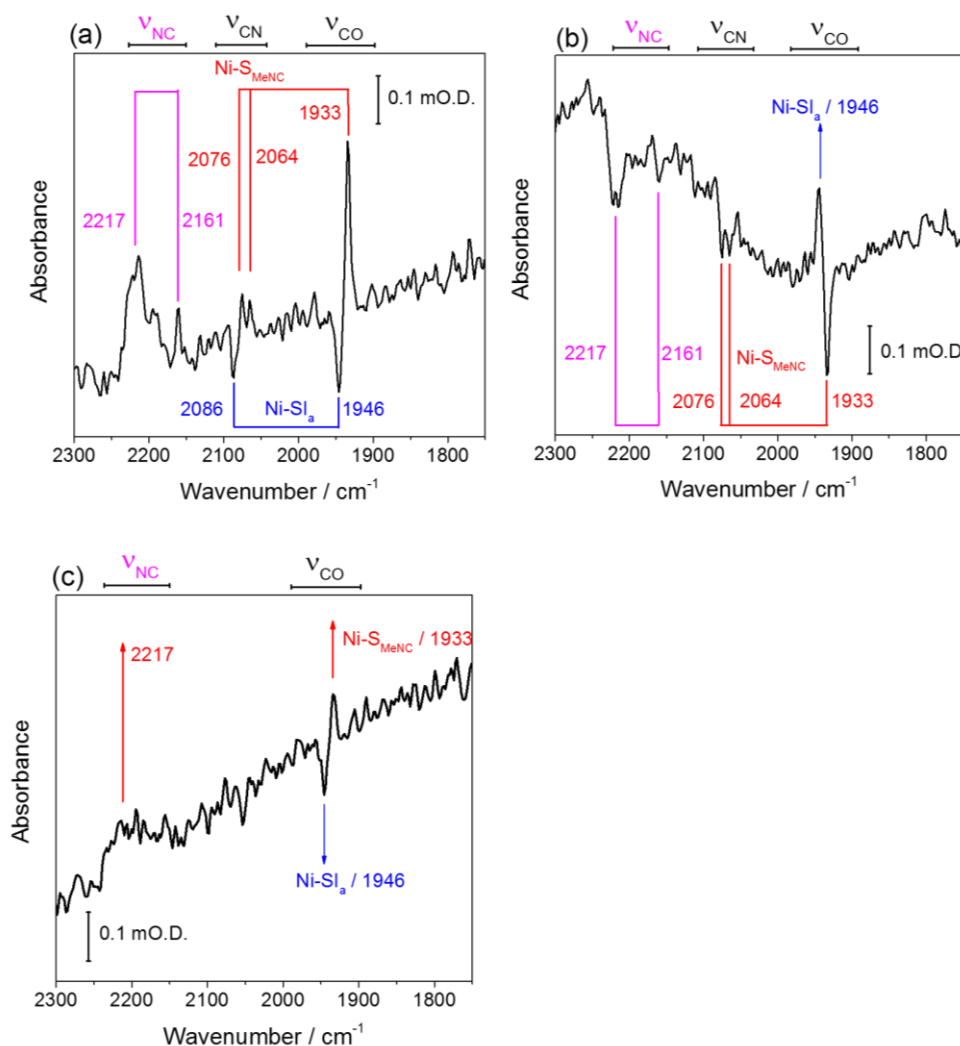


Figure 7-11. ATR-IR difference spectra of Hyd-2 triggered by changes of MeNC concentration at -100 mV. Panel (a): the spectrum was collected in the presence of 2.8 mM MeNC, with a background spectrum recorded before introduction of the inhibitor. Panel (b): the spectrum was recorded after rinsing the cell with clean buffer solution, and then was processed against the one collected with 2.8 mM MeNC present. Panel (c): the difference spectrum was triggered by introduction of 2.8 mM MeNC under 50% CO, with the one collected without MeNC present but also under 50% CO as the background. Other conditions: pH 6 (mixed buffer); 25 °C.

Mutually exclusive binding of CO and MeNC to the active site of Hyd-2

From the results described above, MeNC imposes inhibition on [NiFe]-hydrogenases in a similar way to CO; MeNC inhibits the catalytic activities of both H₂ oxidation and proton reduction, and reacts with Ni-SI_a to form the inhibited species reversibly. Crystallographic studies on the [NiFe]-hydrogenase from *DvMF* showed that CO binds to the Ni of the active site (see Figure 6-1).⁷⁴ Therefore, if the reaction of CO and MeNC with hydrogenases is mutually exclusive, this provides indirect evidence that MeNC might also coordinate to the Ni. Here, Hyd-2 is chosen for examination, as CO serves as a more potent inhibitor to it compared with Hyd-1, i.e. the K_I of H₂ oxidation by CO were determined to be $51 \pm 6 \mu\text{M}$ for Hyd-1 at -0.060 V and $3 \pm 1 \mu\text{M}$ for Hyd-2 at -0.175 V under 20% H₂ (*cf.* Chapter 6).⁶⁷

Figure 7-11, panel c depicts a difference spectrum recorded at -100 mV triggered by introducing 2.8 mM MeNC under 50 % CO (with the spectrum collected without MeNC present but also under 50% CO as the background); the experiment was carried out using the same enzyme particle film as that depicted in panel (a) under comparable conditions. In the presence of 50% CO, introduction of MeNC leads to only little change in species composition at this potential; only a fairly weak negative peak of 1946 cm⁻¹ (ν_{CO}) and a positive peak of 1933 cm⁻¹ (ν_{CO}) are seen. This is in sharp contrast to the observation when CO is absent (*cf.* panel a), in which a significant conversion from Ni-SI_a to Ni-S_{MeNC} occurs. The results imply that CO and MeNC inhibit Hyd-2 competitively.

Discussion

These results demonstrate a reversible reaction between MeNC and [NiFe]-hydrogenases that is revealed in clear detail by PFE and PFIRE spectroscopy. As depicted by chronoamperometric experiments (see Figure 7-2 and 7-4), the introduction of MeNC inhibits the activity of H₂ oxidation for both Hyd-1 and Hyd-2 at -100 mV. The catalytic activity is readily recoverable by simply diluting MeNC in the electrochemical cell. Cyclic voltammetry (see Figure 7-3 and 7-5) indicates that MeNC results in a decrease in H₂ oxidation and proton reduction (for Hyd-2 only) currents regardless of electrode potential. Such a feature implies that MeNC targets a species which is directly involved in the catalytic cycle. This characteristic is in sharp contrast to cyanide (an isoelectronic π -acid ligand with isocyanide), since it only reacts with [NiFe]-hydrogenases in a narrow potential window (*cf.* Figure 5-1), meaning that it interacts with a species which is in rapid equilibrium with active states (i.e. Ni-SI_r state, *cf.* Figure 5-4). Direct evidence of this argument is provided by PFIRE spectroscopy, which clearly shows that Ni-SI_a converts to Ni-S_{MeNC} upon flowing MeNC into the cell at -100 mV for Hyd-1 and Hyd-2, and a reverse spectroscopic feature is seen in the difference spectrum after flushing out the inhibitor. In contrast, a cyanide inhibited species is not observed, except for Hyd-1 at high concentrations of cyanide (*cf.* Figure 5-6). PFIRE studies also show that CO and MeNC reacts with Hyd-2 competitively, providing strong evidence that MeNC also coordinates to the Ni when it burrows into the active site. Aforementioned observations indicate that MeNC inhibits [NiFe]-

hydrogenases in a similar way to CO, which is foreseeable because both of them are neutral π -acid ligands.

Generally, among isocyanide complexes, when isocyanides are coordinated to zero-valence metals, the bonding between the isocyanide and the transition metal is more weighted toward π -back donation from the metal, and thus the $\text{N}\equiv\text{C}$ stretching band is shifted to a lower frequency.¹³⁴ In contrast, when isocyanides are coordinated to monopositive or dipositive metal ions, the inductive effect is more pronounced in coordination, with little or no back donation from metals. And thus the infrared absorption of the isocyano group is shifted to a higher wavenumber.¹³⁴ However, the investigation of bound MeNC with infrared absorption downward shift by *ca.* 30 cm^{-1} relative to the free aqueous MeNC for both Hyd-1 and Hyd-2 is not in line with the general feature of synthetic isocyanide complexes. A possible reason for this investigation might be that MeNC is not ligated to the active site in a linear fashion due to the steric constraint within the catalytic pocket. This argument would be in good agreement with other well-established isocyanide adducts of metalloproteins revealed by crystallography, such as *n*-butyl isocyanide-bound P450cam,¹³⁵ and ethyl isocyanide-bound myoglobin,¹³⁶ and [Fe]-hydrogenase/isocyanide complexes.¹²⁶ Moreover, studies of the CO-inhibited [NiFe]-hydrogenase from *DvMF* also found that the exogenous CO binds to the Ni in a bent conformation⁷⁴ (see Figure 6-1).

7.3 More bulky isocyanides

Isocyanides with longer alkyl chain, i.e. ethyl-, *n*-butyl, and *t*-butyl-, were used in this section as probes into the steric constraints within the catalytic pocket of Hyd-1.

The inhibitory effects of more bulky isocyanides on Hyd-1 were ascertained by chronoamperometry on a RDE first; the inhibitors were introduced into the cell in contact with the enzyme film at -100 mV under 100% H₂ for ethyl isocyanide, and 10% H₂ for *n*-butyl and *t*-butyl isocyanides (Figure 7-12). In sharp contrast to MeNC, much higher concentrations are required (at least 100 mM) to see the inhibitory effects on H₂ oxidation by all of these isocyanides with a longer tail. This might be attributed to the poor solubility of these organic compounds, and thus more of these inhibitors has to be used to make more emulsions (assisted by a fast rotation rate of the RDE to form a vortex) for having an effect on the immobilised Hyd-1 film. Under 100% H₂, introducing aliquots of ethyl isocyanide solution to give a final concentration of 260 mM in the cell at -100 mV causes loss of *ca.* 8 % of activity in H₂ oxidation for Hyd-1 (panel a). The slight inhibition is attributed to the observation that only little Ni-SI_a species is present at this potential to be attacked by ethyl isocyanide, when Hyd-1 is turning over under 100% H₂, as happens for CO inhibition (*cf.* section 6.3). A lower H₂ concentration (10% H₂) is used to examine the effects of *n*-butyl (panel b) and *t*-butyl (panel c) isocyanides. Losses of activity by *ca.* 67% and 12% were investigated imposed by *n*-butyl (117 mM) and *t*-butyl (108 mM)

isocyanides, respectively. The slight recovery in currents after injections of these inhibitors is attributed to the reactivation from Ni-B, due to trace O_2 present in the neat isocyanide stock solutions.

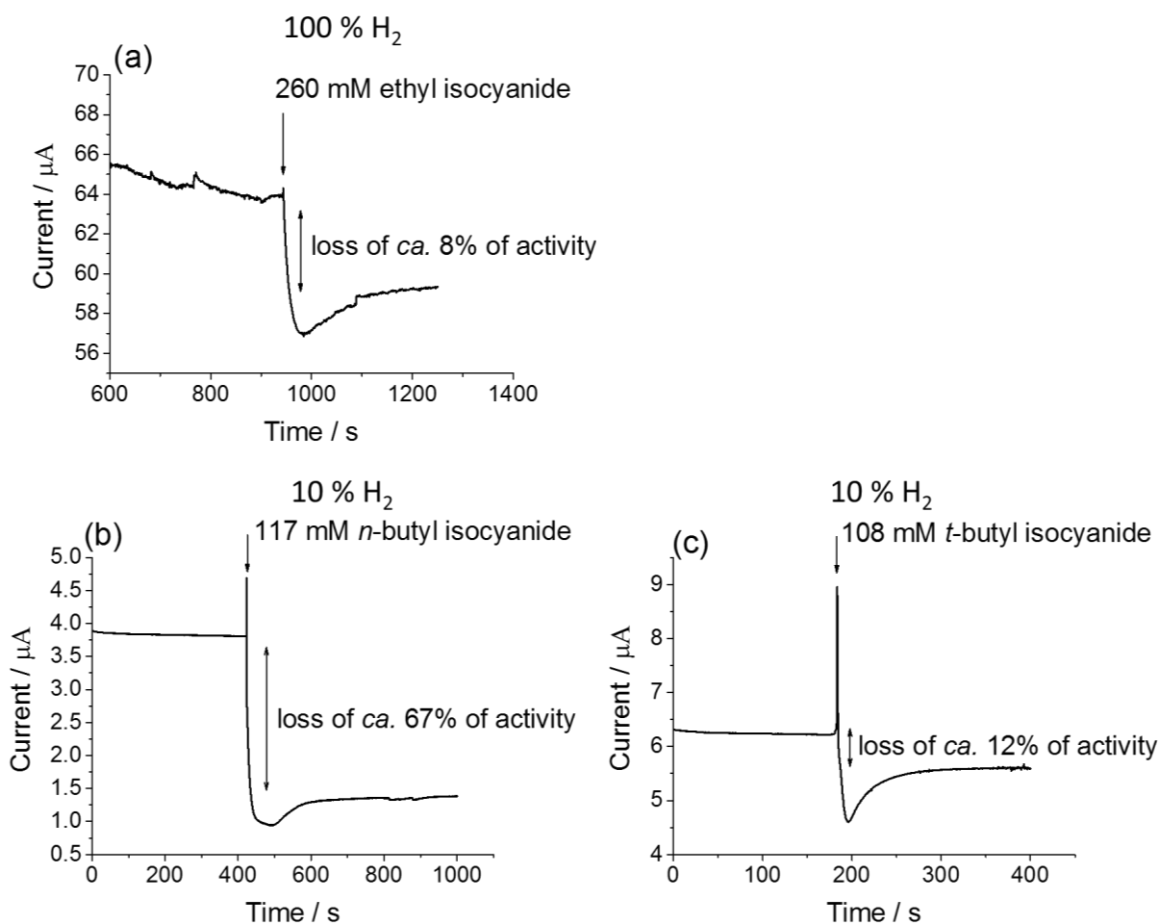


Figure 7-12. Chronoamperometry traces of immobilised Hyd-1 film on a RDE at -100 mV. Aliquots of ethyl isocyanide (a), *n*-butyl isocyanide (b), and *t*-butyl isocyanide (c) were injected to give final concentrations in the cell of 260 mM, 117 mM and 108 mM at time points indicated by arrows, respectively. The experiments were carried out under 100% H_2 for ethyl isocyanide, and 10% H_2 for *n*-butyl and *t*-butyl isocyanides. Other conditions: pH 6 (mixed buffer); 28°C; electrode rotation rate, 3000 rpm.

PFIRE was then used to provide structural evidence of how these bulky isocyanides affect Hyd-1 under 100% Ar. Figure 7-13 depicts ATR-IR difference spectra of Hyd-1, triggered by changes of concentration of (a) ethyl isocyanide, and (b) *n*-butyl isocyanide at -100 mV. Introduction of ethyl isocyanide to give a final concentration of 277 mM in contact with the Hyd-1 particle film leads to a conversion from Ni-SI_a to Ni-S_{EtNC} (panel a, black trace), as shown by characteristic peaks of ν_{CO} at 1929 cm⁻¹ (in the negative direction) and 1915 cm⁻¹ (in the positive direction). The split of free ethyl isocyanide bands, ν_{NC} , at a region of 2300 - 2100 cm⁻¹, is contributed by Fermi resonance (*vide supra*, see section 7.1) with the band at 2149 cm⁻¹ arising from the isocyano group trapped in the micelle-like droplets. The inhibition imposed by ethyl isocyanide is also readily reversible; flushing out ethyl isocyanide from the spectroelectrochemical cell gives a reverse feature in the difference spectrum (panel a, red trace), a positive peak at 1929 cm⁻¹ and a negative band at 1915 cm⁻¹, corresponding to a recovery of the catalytic state, Ni-SI_a.

n-butyl isocyanide with a longer alkyl chain has a similar effect on Hyd-1, as depicted in panel (b). At -100 mV, an injection of *n*-butyl isocyanide stock solution to give a final concentration of 115 mM in the cell leads to the formation of Ni-S_{*n*-butyl NC}, which has the endogenous ν_{CO} at 1914 cm⁻¹, accompanied by losing Ni-SI_a state. Three strongly absorbing bands at 2196, 2171 and 2147 cm⁻¹ are seen in the region of free isocyanide vibrational band (ν_{NC}), 2300 - 2100 cm⁻¹. These three peaks are not contributed by the Fermi resonance, as this effect is unique to the ethyl group among alkyl isocyanides,

and the reason is not clear (*vide supra*, see section 7.1).¹²⁹ The band at 2147 cm^{-1} also arises from the isocyano group in micelle-like droplets, due to its poor solubility in water. The peak at 2171 cm^{-1} is the $\text{N}\equiv\text{C}$ stretching band of *n*-butyl isocyanide in aqueous form.¹²⁹ However, the origin of the band at 2196 cm^{-1} is unclear; this peak alone appears in a control experiment when the BP2000 carbon nanoparticles are used to make a film (without immobilised enzyme) in contact with the isocyanide solution. Thus, this band might result from an interaction between *n*-butyl isocyanide and the BP2000 particles. This interaction is not studied further in this Thesis, as it does not affect the isocyanide inhibition of Hyd-1 and data interpretation.

A more bulky isocyanide with the R group of *t*-butyl was also used to probe how spacious the catalytic pocket of Hyd-1 is. However, the coordination of this isocyanide to the active site is not seen in the FTIR spectra under the current conditions (100% Ar without the substrate present; -100 mV). The features in the FTIR spectra corresponding to interconversions from Ni-R to Ni-C, Ni-SI_a and Ni-B as potentials were stepped to more positive values are still present (and *vice versa*) without an observation of extra peaks from an inhibited species, even at an extremely high concentration of 300 mM *t*-butyl isocyanide (*data not shown*).

The possible reason why no inhibited species is observed for *t*-butyl isocyanide is thought as follows: only tiny amounts of *t*-butyl isocyanide are able to reach the active site due to high steric hindrance when a compound bearing a bulky R group burrows into the active site via the hydrophobic gas channel. Thus, the

concentration of the inhibited species, i.e. Ni-S-*t*-butyl NC, is not high enough to be resolved by FTIR. The much poorer solubility in water of this compound is also a contributory factor. This investigation in FTIR spectroscopy is in line with the result from chronoamperometry on a RDE: only a little oxidation current (*ca.* 12 % of activity) is lost upon introduction of *t*-butyl isocyanide (108 mM) with a low concentration of the substrate present, 10% H₂, compared with that imposed by *n*-butyl isocyanide (*cf.* Figure 7-12, panel b, *ca.* 67% of activity is inhibited by 117 mM inhibitor under 10% H₂).

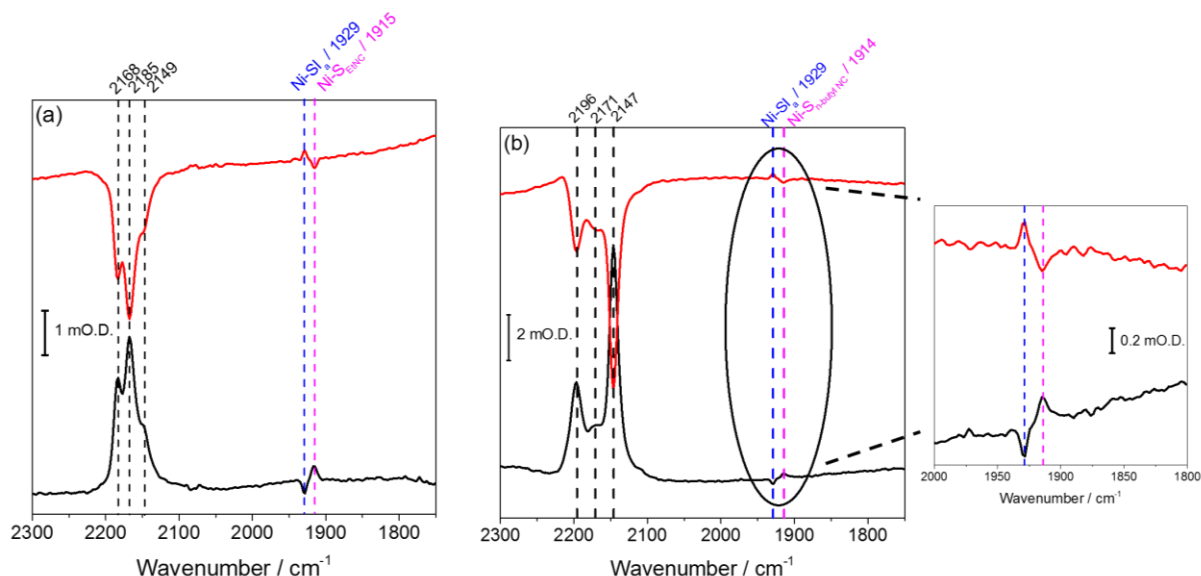


Figure 7-13. ATR-IR difference spectra of Hyd-1 at -100 mV triggered by changes in concentration of (a) ethyl isocyanide and (b) *n*-butyl isocyanide. Black trace: the spectra were collected in the presence of 277 mM ethyl isocyanide / 115 mM *n*-butyl isocyanide, with a background spectrum recorded before the introduction of an inhibitor. Red trace: the spectra were recorded after rinsing the cell with clean buffer solution, and then were processed against the one collected with the inhibitor present. Other conditions: pH 6 (mixed buffer); 25 °C.

Discussion and summary

The results obtained in this section show that isocyanides with longer alkyl chains, ethyl- and *n*-butyl, are also able to inhibit Hyd-1 reversibly. This implies that the catalytic pocket of Hyd-1 is more spacious than we thought before, and thus bigger organic molecules are capable of getting in and out freely. This finding could open up interesting further projects to see if [NiFe]-hydrogenases can perform other reactions on organic molecules, such as hydrogenation. A similar concept that hydrogenases serve as a promising catalyst to perform hydrogenation on organic molecules has been proposed from the work of Foster *et al.* in studies of formaldehyde inhibition of [FeFe]-hydrogenases.¹³⁷ The formaldehyde was suggested most likely to target the Fe-hydrido (Fe-H) intermediate by DFT, displaying a close similarity to Noyori catalyst for hydrogenation of ketones, aldehydes, and imines,^{138,139} albeit the expected product of this reaction, methanol, was not detected in their study.

Chapter 8: Conclusions and outlook

In this Thesis, a modified particle-electrode is developed to have a high loading of Hyd-2, and therefore studies of Hyd-2 under catalytic turnover using the surface FTIR technique of PFIRE is possible. Using the approach of PFIRE, *in situ* studies of reactions of Hyd-1 and Hyd-2 with a series of π -acid ligands (cyanide, CO and isocyanides) are carried out systematically under precise potential control for comparison.

Development of a particle electrode with high Hyd-2 loading

A surfactant-modified particle electrode (with high surface area) has been designed and demonstrated as a good approach to have high loading of Hyd-2 for use in PFIRE. The high loading of Hyd-2 is facilitated by hydrophobic interactions, by which Hyd-2 is able to adsorb on the modified particle-electrode in the right orientation for effective interfacial electron transfer between the active sites of enzymes and electrode surfaces without causing significant overpotential in hydrogen cycling. This is in contrast to attempted covalent attachment approach, which leads to significant overpotential for H₂ oxidation by Hyd-2.

Insights into hydrogenase mechanism

After the demonstration of the successful strategy for constructing a particle electrode with high Hyd-2 loading, redox characterisation by PFIRE was

performed for Hyd-2 (which was not fully characterised previously). The experiments were carried out under turnover and non-turnover conditions. The results show that states of Ni-SI_a, Ni-C and Ni-R are always present (with the concentration of Ni-SI_a greater or similar to that of Ni-R), when Hyd-2 is turning over for H₂ oxidation and proton reduction. This implies that the proton reduction might be performed through the same elementary processes as H₂ oxidation but in a reverse direction. The precise detail of the mechanism might be different for proton reduction, but the principal 'equilibrium' states involved are the same as H₂ oxidation. The distribution of states for Hyd-2 when it is turning over under H₂ is distinct to that of Hyd-1. For Hyd-1, Ni-R is always present at far greater level than Ni-SI_a during catalytic H₂ oxidation, and Ni-L is also present. The differences can be rationalised by considering the unusually high redox potentials of the FeS clusters equipped in the O₂-tolerant MBHs, such as Hyd-1. This factor leads to distinct kinetics in the elementary process for the conversion from Ni-C to Ni-SI_a, stabilising Ni-L as a detectable intermediate, where electron occupancy of the proximal FeS cluster is likely to impede oxidation of Ni-L to Ni-SI_a. In Hyd-2, there is either a “concerted” proton and electron transfer step to take Ni-C to Ni-SI_a or Ni-L is oxidised so rapidly when it forms that it is not detected in PFIRE experiments.

Studies of reactions of Hyd-1 and Hyd-2 with a series of π -acid ligands

A common inhibitor of metalloproteins, cyanide, was studied for its effect on Hyd-1 and Hyd-2 in this Thesis, as there are few reports addressing the interaction between cyanide and [NiFe]-hydrogenases, and a clear picture of this reaction has not been reported.

The anionic σ -donor and π -acid ligand, cyanide, was found to be able to promote formation of the ready state, Ni-B, which is otherwise formed slowly without O₂.

Hyd-1 and Hyd-2 were found to be similar in their responses to CO compared to other [NiFe]-hydrogenases studied previously: CO attacks the active site at the Ni-SI_a level. Moreover, benefitted by the technique, PFIRE, used in this study, it was possible to tackle the puzzling feature that the O₂-tolerant MBHs are more resistant to CO when they are turning over under H₂ compared with standard hydrogenases. It seems that the main reason contributing to this feature is that the state which can be attacked by CO, i.e. Ni-SI_a, is only present in a relatively small amount under turnover conditions in O₂-tolerant Hyd-1, as the majority species is always the Ni-R state. Thus, the interference imposed by CO to the dynamic equilibrium of active states during catalytic turnover is smaller compared to the standard hydrogenase, Hyd-2.

Isocyanides were found to inhibit Hyd-1 and Hyd-2 in a similar way to CO. The use of more bulky isocyanides as probes reveals that the catalytic pocket of

Hyd-1 is spacious, opening up opportunities for seeking other types of reactions on organic molecules catalysed by the hydrogenase active site.

Concluding remarks

A systematic study of how Hyd-1 and Hyd-2 react with a series of π -acid ligands, cyanide, CO and isocyanides is described in Chapter 4–7. The results reveal that the O₂-tolerant MBH, Hyd-1, and the standard hydrogenase, Hyd-2, are similar in their responses to these ligands except that Hyd-1 is less sensitive to inhibition by CO and isocyanides than Hyd-2 (*ie.* it has a higher K_I).

It is well-known that these two types of hydrogenases have a distinct feature in reaction with the π -acid ligand O₂. The FeS clusters equipped in the O₂-tolerant MBHs have unusually high redox potentials, which are able to supply electrons rapidly at more oxidising conditions to the active site. One theory put forward to explain the O₂ tolerance of Hyd-1 is that the O₂ can be reduced quickly to the hydroxide species when it attacks the active site under catalytic turnover. The hydroxide species is then trapped at the bridging position in the bimetallic centre to form the Ni-B state, which can be reactivated rapidly to bring the enzyme back to the catalytic cycle under reducing conditions. However, the FeS clusters accommodated in standard hydrogenases do not have the compatible redox potentials to reduce O₂ under the same conditions with O₂-tolerant MBHs.

The work on CO and isocyanides in this Thesis suggests that the amount of Ni-SI_a present at each potential during catalytic turnover may also be important

in controlling the reaction with π -acid ligands. The low amount of Ni-SI_a for Hyd-1 during catalytic turnover would also contribute to its O₂ tolerance.

Outlook and future work

Protein film infrared electrochemistry (PFIRE) has been demonstrated as a powerful tool for hydrogenase studies in this Thesis. The development of a modified particle-electrode for Hyd-2 for use in PFIRE in this Thesis enables a detailed study on it for comparison with the O₂-tolerant MBH, Hyd-1. The further goal of exploiting PFIRE on these enzymes would be to catch the transient intermediates when the hydrogenase is turning over under the substrates (H₂ or H⁺) by proper experimental designs, such as to reduce the rate of H₂ cycling by carrying out experiments at low temperatures with heavy substrates, i.e. under D₂ in D₂O, or preparing genetic variants with slower turnover rates. This could address the important question of whether Ni-L is an intermediate during catalysis by O₂-sensitive hydrogenases, such as Hyd-2.

Studies of isocyanides with Hyd-1 reveal that there is a spacious room within the catalytic pocket. Therefore, other organic compounds can be used to examine if the hydrogenase active site is able to catalyse other reactions, and the product formation can be monitored by PFIRE *in situ*.

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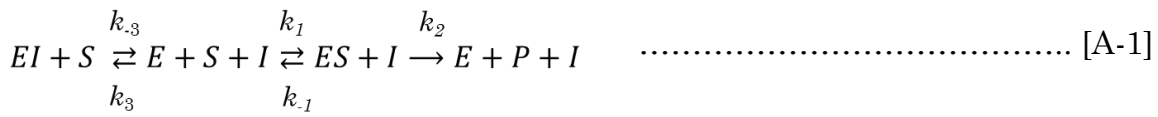
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Appendix: Derivation of the competitive inhibition constant⁹¹

A binding of the competitive inhibitor (I) to the free enzyme (E) is introduced into a single-substrate enzyme system, [2-16], which gives a modified model expressed in [A-1]:



Applying the steady-state approximation to [A-1], which means the concentration of each enzyme species is not changing, [A-2] is derived:

$$\frac{d[EI]}{dt} = \frac{d[ES]}{dt} = \frac{d[E]}{dt} = 0 \quad \text{..... [A-2]}$$

$$\frac{d[EI]}{dt} = 0 \Rightarrow k_{-3}[EI] = k_3[E][I] \quad \text{..... [A-3]}$$

$$\frac{d[ES]}{dt} = 0 \Rightarrow k_1[E][S] = (k_{-1} + k_2)[ES] \quad \text{..... [A-4]}$$

Rearranging [A-4] gives $[E] = \frac{(k_{-1}+k_2)[ES]}{k_1[S]}$, where $\frac{k_{-1}+k_2}{k_1}$ can be replaced by the Michaelis constant, K_M ([2-19]).

Therefore,

$$[E] = \frac{K_M[ES]}{[S]} \quad \text{..... [A-5]}$$

Substituting [A-5] into [A-3] and rearranging gives

$$[EI] = \frac{K_M k_3 [ES][I]}{k_{-3}[S]} \dots\dots\dots [A-6]$$

The dissociation constant of the EI complex, K_I , is defined as $\frac{k_{-3}}{k_3}$, and therefore

$[EI]$ can be expressed as follows:

$$[EI] = \frac{K_M [ES][I]}{K_I [S]} \dots\dots\dots [A-7]$$

The total concentration of the enzyme, E_T , can be expressed as follows:

$$\begin{aligned} [E]_T &= [E] + [EI] + [ES] = \frac{K_M [ES]}{[S]} + \frac{K_M [ES][I]}{K_I [S]} + [ES] \\ &= [ES] \left(\frac{K_I K_M + K_M [I] + K_I [S]}{K_I [S]} \right) \end{aligned}$$

Therefore, $[ES]$ can be expressed as follows:

$$[ES] = \left(\frac{K_I [S]}{K_I K_M + K_M [I] + K_I [S]} \right) [E]_T \dots\dots\dots [A-8]$$

Finally, the catalytic rate, v , can be written in [A-9]:

$$v = \frac{k_2 K_I [S][E]_T}{K_I K_M + K_M [I] + K_I [S]} = \frac{k_2 [S][E]_T}{K_M \left(1 + \frac{[I]}{K_I} \right) + [S]} = \frac{v_{max} [S]}{K_M \left(1 + \frac{[I]}{K_I} \right) + [S]} \dots\dots\dots [A-9]$$