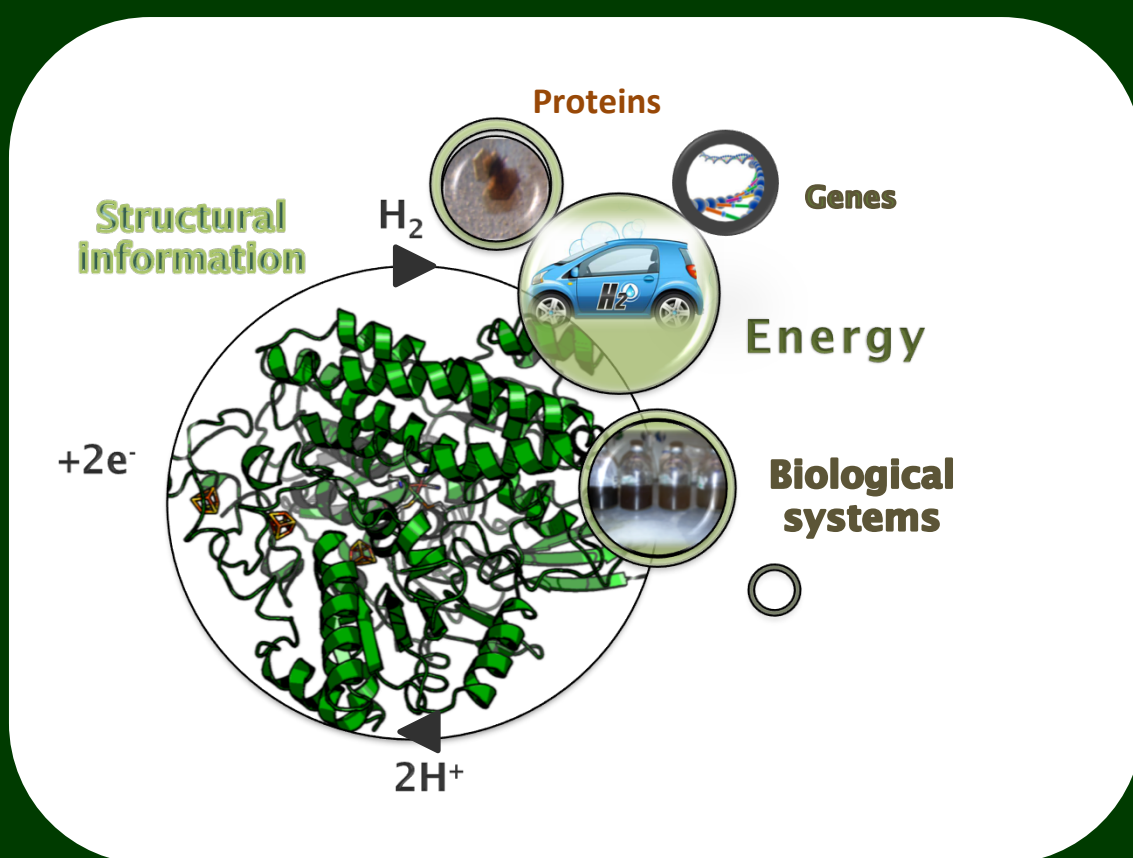


Structural and functional studies of a high activity NiFeSe Hydrogenase

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Knowledge Creation



"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence."

Louis Pasteur



17th December 2014

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Cristina Gutiérrez-Sánchez, David Olea, Marta C Marques, Victor M Fernández, Inês A C Pereira, Marisela Vélez, Antonio L De Lacey. 2011. Oriented immobilization of a membrane-bound hydrogenase onto an electrode for direct electron transfer, *Langmuir*, 27 (10): 6449-57

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Resumo

A consciência de que os combustíveis fósseis existem em quantidades limitadas tem estimulado a investigação na área da produção de energia a partir de fontes renováveis. No futuro, as fontes de energia devem ser abundantes e com baixo impacto no meio ambiente. O hidrogénio surge com potencial para satisfazer estes requisitos. No entanto, os métodos atuais de produção de H_2 dependem de recursos não-renováveis. A produção biológica de H_2 a partir de luz solar ou de biomassa são alternativas aliciantes.

As Hidrogenases (Hases) são os catalisadores biológicos responsáveis pela interconversão reversível de H_2 em prótons e electrões. Estas metaloenzimas têm sido alvo de pesquisa há mais de 80 anos. Esta pesquisa resultou na elucidação da sua estrutura, do seu mecanismo catalítico e do seu processo de maturação. As Hases podem, potencialmente, vir a ser usadas como catalisadores de oxidação de H_2 em células de combustível ou em dispositivos fotocatalíticos para a produção de H_2 . As Hases são classificadas de acordo com a composição metálica do seu centro ativo e de acordo com a presença/ausência de centros de [Fe-S]. Podem ser classificadas como Hases de NiFe, FeFe ou apenas Fe. Em algumas Hases de NiFe, uma das cisteínas que está ligada ao níquel no centro ativo, pode ser substituída por uma selenocisteína. Com base na sua semelhança de sequências, ambas as Hases de NiFe e NiFeSe são consideradas como um único grupo.

Nesta tese, o organismo modelo escolhido foi a bactéria redutora de sulfato *Desulfovibrio vulgaris* Hildenborough (DvH), uma bactéria anaeróbia estrita que é geralmente encontrada em ambientes anóxicos. Sabendo que o genoma de DvH codifica para sete Hases, este fato torna este organismo um

bom modelo para estudar esta classe de enzimas. A Hase selecionada foi a Hase de NiFeSe, que possui uma atividade catalítica muito elevada e foi investigada através de uma combinação de abordagens bioquímicas, biofísicas e estruturais.

O *background* científico deste trabalho de pesquisa começa por ser abordado no capítulo 1.

No capítulo 2, foi investigada a influência da estrutura da Hase de NiFeSe que rodeia o centro ativo. Foi executado um estudo teórico e experimental avaliando a atividade catalítica de troca H/D por espectrometria de massa em combinação com estudos de simulação por dinâmica molecular. Ambos os resultados experimentais e computacionais consideram a existência de um "efeito tipo *cage*" dos resíduos que circundam o centro ativo, modulando a assim a função catalítica desta enzima.

No capítulo 3, foram analisadas as estruturas cristalográficas de alta resolução da forma solúvel da NiFeSe Hase em três estados *redox* diferentes. Esta investigação elucida as alterações estruturais que ocorrem no centro ativo da enzima durante os processos de redução e re-oxidação, revelando o papel importante do centro de [4Fe-4S] proximal, neste processo.

No capítulo 4 é descrita a implementação de um sistema para a expressão homóloga da Hase solúvel de NiFeSe de *DvH*. A nova Hase recombinante foi então caracterizada e mostrou-se funcionalmente semelhante à proteína nativa. A produção de Hases NiFeSe recombinantes enceta o caminho para a engenharia de enzimas com propriedades catalíticas otimizadas. Como prova de conceito, foi criada uma forma mutada da proteína onde a selenocisteína presente no centro ativo foi substituída por uma cisteína, convertendo assim a Hase de NiFeSe numa enzima de NiFe. Desta forma, foi

possível avaliar o papel deste resíduo na elevada atividade catalítica e na tolerância razoável ao oxigénio exibida pelas Hases de NiFeSe. Várias estruturas cristalográficas de alta-resolução da forma mutante da Hase de NiFeSe foram obtidas e é descrito ainda um ensaio *in vitro* que reconstitui em parte o centro ativo de Ni-Fe, consequência da incorporação do níquel ser deficiente neste mutante (capítulo 4). A mutação de selenocisteína para cisteína converteu a proteína numa enzima mais vulnerável ao ataque do O₂ e com uma actividade catalítica muito inferior à descrita para a Hase de NiFeSe recombinante. Esta investigação permitiu novos *insights* sobre as propriedades bioquímicas e estruturais das Hases de NiFeSe, bem como a análise das vias de inativação pelo O₂, deste subgrupo de Hases.

No futuro, o trabalho a desenvolver passará por completar a caracterização da Hase de NiFeSe recombinante e produção de novas variantes, recorrendo também a outras técnicas espectroscópicas e eletroquímicas, a fim de desenvolver o conhecimento acerca destas metaloenzimas. Esta investigação, terá sempre como objetivo contribuir para a sua implementação e aplicação em sistemas biotecnológicos. A continuidade deste trabalho de pesquisa ajudará certamente na concepção de catalisadores de H₂ com propriedades optimizadas recorrendo a Hases com uma maior tolerância ao O₂.

ABSTRACT

The awareness that fossil fuels exist in limited quantities has stimulated research into energy production from renewable sources. Future energy sources should be plentiful with negligible impact on the environment. Hydrogen has the potential to satisfy these requirements. Nevertheless, current methods of H₂ production rely on non-renewable resources. Biological H₂ production from sunlight or biomass is an appealing alternative to current production methods.

Hydrogenases (Hases) are the biological catalysts responsible for the reversible interconversion of H₂ into protons and electrons. These metalloenzymes have been the subjects of research for more than 80 years, which resulted in the elucidation of their structure, catalytic mechanism, and maturation factors. Hases can potentially be used as H₂ oxidation catalysts in fuel cells or for H₂ production by photocatalytic devices. Hases are classified according to the metal composition of their active site and the presence/absence of Fe-S clusters: NiFe, FeFe, and Fe-only. In some NiFe Hases, one of the Ni-bound cysteine (Cys) residues is replaced by selenocysteine (Sec). On the basis of sequence similarity, the NiFe and NiFeSe Hases can be considered a single group.

In this thesis, the model organism chosen was the sulphate reducing bacterium *Desulfovibrio vulgaris* Hildenborough (DvH), a strictly anaerobic bacterium that is generally found in anoxic environments. The DvH genome codes for seven Hases, making this organism a good model to study this enzyme class. The Hase selected was the highly active NiFeSe Hase, and it was studied by a combination of biochemical, biophysical, and structural approaches.

The scientific background of this research work is summarized in chapter 1.

In chapter 2 the influence of the protein structure surrounding the active site in NiFeSe Hases was investigated. An experimental and theoretical study of the catalytic activity of a NiFeSe Hase has been performed by H/D exchange mass-spectrometry and molecular dynamics (MD) simulations. Both experimental and computational results are in accordance with a “cage effect” of the residues that encircle the active site, modulating its catalytic function.

In chapter 3, high-resolution X-ray crystallographic 3D structures of the NiFeSe Hase native soluble form in three different redox states are reported. This study highlighted the structural changes that take place at the active site during enzyme reduction and re-oxidation, revealing the role of the proximal [4Fe-4S] cluster in this process.

In chapter 4 a homologous system for expression of the soluble NiFeSe Hase from *DvH* is described. The recombinant Hase was characterized and shown to be functionally similar to the native protein. The production of recombinant NiFeSe Hases paves the way for engineering enzymes with optimized catalytic properties. As a proof of concept, a mutated form of the protein was created where the active site Sec residue was replaced by a Cys, effectively converting the NiFeSe Hase into a NiFe enzyme. The purpose of this mutation was to assess the role of the Sec residue in the high catalytic activity and in the reasonable O₂-tolerance displayed by the NiFeSe Hases. Several high-resolution X-ray crystallographic structures of the NiFeSe Hase mutant form are reported and an *in vitro* assay was developed that partly reconstituted the Ni-Fe active site, very poorly incorporated in the untreated Cys mutant (chapter 4). The Sec to Cys mutation converted the protein into an enzyme more vulnerable

to O₂ attack and with lower catalytic activity when compared with the recombinant NiFeSe Hase. These studies provided new insights into the biochemical and structural properties of NiFeSe Hases, as well as into the O₂-inactivation pathways of this Hase subgroup.

Future work will be needed to characterize the recombinant NiFeSe Hase and produce new mutant variants, which will be characterized by other spectroscopic and electrochemical techniques, in order to further expand our understanding of these metalloenzymes with the aim to implement their practical applications. The continued research work will certainly contribute to the design of H₂ catalysts with desired properties and improved O₂-tolerance.

ABBREVIATIONS

<i>Aa</i>	<i>Aquifex aeolicus</i>
<i>Av</i>	<i>Allochromatium vinosum</i>
C/Cys	Cysteine(s)
Cv	Cyclic voltammograms
cyt c	cytochrome c type-I
<i>D.</i>	<i>Desulfovibrio</i>
DDM	Dodecyl-xx-maltoside
<i>Df</i>	<i>Desulfovibrio fructosovorans</i>
<i>Dg</i>	<i>Desulfovibrio gigas</i>
<i>DvH</i>	<i>Desulfovibrio vulgaris</i> Hildenborough
<i>DvMF</i>	<i>Desulfovibrio vulgaris</i> Miyazaki F
Dwn	Downstream
E_0^0	Standard redox potential
<i>E. coli</i>	<i>Escherichia coli</i>
EPR	Electron paramagnetic resonance
Exp Vctr	Expression vector
FeFe Hase	Iron-iron hydrogenase
[FeS]	Iron-sulphur cluster
FTIR	Fourier transform infra-red
Fw	Forward
GC	Gas Chromatography
Hase	Hydrogenase
HynAB	NiFe hydrogenase
<i>Hm</i>	<i>Hydrogenovibrio marinus</i>

Hys	NiFeSe hydrogenase
HysAB	NiFeSe hydrogenase
HOPG	Highly oriented pyrolytic graphite
ICP	Inductively coupled plasma resonance
IR	Infra-red
kDa	kilodalton
<i>M.</i>	<i>Methanococcus</i>
MV	Methyl-viologen
NiFe Hase	Nickel-iron hydrogenase
NiFe ₁ Hase	Nickel-iron hydrogenase isoenzyme -1
NiFeSe	Nickel-iron–selenium hydrogenase
PDB	Protein Data Bank
PEG	Polyethylene glycol
PGE	Pyrolytic graphite edge
<i>R. capsulata</i>	<i>Rhodopseudomonas capsulata</i>
<i>Re</i>	<i>Ralstonia eutropha</i> H16
Rev	Reverse
RT	Room temperature
SDS	Sodium dodecyl sulfate
Sec/U	Selenocysteine
spp.	species
SRB	Sulphate Reducing Bacteria
<i>T.</i>	<i>Thermodesulfovibrio</i>
Tris	Tris (hydroxymethylaminomethane)
<i>wt</i>	wild type

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
LIST OF PUBLICATIONS.....	v
RESUMO.....	vii
ABSTRACT.....	x
ABBREVIATIONS.....	xiii
CHAPTER 1	1
1.1 HYDROGEN, A SUSTAINABLE ENERGY CARRIER	2
1.2 BIOLOGICAL HYDROGEN PRODUCTION	3
1.3 HYDROGEN UTILIZATION BY SRB.....	4
1.4 HYDROGENASES	11
1.5 NIFE HYDROGENASES	15
1.5.1 NiFe Hases maturation.....	17
1.5.2 Tat Protein Export Pathway	21
1.5.3 The catalytic cycle of NiFe Hases	22
1.5.4 O ₂ -tolerant NiFe Hases.....	26
1.6 NiFeSe Hases	29
1.6.1 Spectroscopic studies.....	32
1.6.2 Structural studies	37
1.6.1 O ₂ tolerance and applications.....	42
REFERENCES	43
CHAPTER 2	55
2.1 INTRODUCTION	57
2.2 MATERIALS AND METHODS	60
2.2.1 Hase purification and preparation	60
2.2.2 Hydrogen/deuterium exchange activity	60

2.2.3	Molecular dynamics (MD) simulations	62
2.3	RESULTS.....	63
2.3.1	Molecular dynamics (MD) simulations	70
2.4	DISCUSSION	72
	ACKNOWLEDGMENTS	77
	REFERENCES	77

CHAPTER 3	81
3.1 INTRODUCTION	83
3.2 MATERIALS AND METHODS	87
3.2.1	Protein production, purification and enzymatic activity assay.....87
3.2.2	Crystallization and X-ray diffraction data collection 88
3.2.3	Structure determination and refinement 92
3.2.4	Sequence analysis tools 96
3.3 RESULTS AND DISCUSSION.....	96
3.3.1	Purification and enzymatic activity 97
3.3.2	Structure of <i>DvH</i> NiFeSe Hase..... 101
3.3.3	The structure of the active site in the different redox forms . 101
	<i>An oxidized cysteine is present in the active site</i>110
	<i>The proximal iron-sulphur cluster</i>112
3.4 CONCLUSIONS..	121
	REFERENCES
3.5 SUPPLEMENTARY RESULTS.....	133

CHAPTER 4	141
4.1 INTRODUCTION	143
4.2 MATERIAL AND METHODS	145
4.2.1	Genetic constructions 145
4.2.2	Cell growth 147
4.2.3	Purification of IPMM01 NiFeSe Hase 147
4.2.4	Construction of IPMM02 U489C 148
4.2.5	Purification of IPMM02 U489C 149

4.2.6	Analytical methods	149
4.2.7	Western blot analysis.....	150
4.2.8	H ₂ production by Gas Chromatography	150
4.2.9	Hase uptake activity assays.....	151
4.2.10	Hydrogen/deuterium exchange activity	151
4.2.11	<i>In vitro</i> metal reconstitution of IPMM02 U489C Hase.....	152
4.2.12	FTIR spectroscopy and FTIR spectroelectrochemical experiments	153
4.2.13	Crystallization and X-ray diffraction data collection	153
4.2.14	Structure determination and refinement	157
4.2.15	Electrochemical studies	159
4.3	RESULTS AND DISCUSSION.....	160
4.3.1	Isolation of IPMM01 NiFeSe Hase.....	160
4.3.2	Isolation of IPMM02 U489C.....	163
4.3.3	The crystal structure of IPMM01-A, the aerobically purified IPMM01 NiFeSe Hase.....	165
4.3.4	The high-resolution structure of IPMM01-N NiFeSe Hase...	170
4.3.5	The impact of Se substitution in the structure of the U489C Hase.....	175
4.3.6	Reconstitution of the Ni-Fe active site in the IPMM02 U489C Hase.....	180
4.3.7	Enzymatic activities.....	186
4.3.8	FTIR spectroscopy	189
4.3.9	O ₂ inactivation	192
4.3.10	Electrochemical studies of the DvH NiFeSe Hase covalently bound to a 4-ATP gold rotating electrode	198
4.3.11	Electrochemical studies of the IPMM01 NiFeSe Hase and IPMM02 U489C on HOPG electrodes	200
4.4	FINAL CONCLUSIONS.....	201
	REFERENCES	202

CHAPTER 5.....207
CONCLUDING REMARKS208
APPENDIX.....211

CHAPTER 1

Introduction

Parts of this chapter were published in:

Inês A. Cardoso Pereira, Ana Raquel Ramos, Fabian Grein, Marta Coimbra Marques, Sofia Marques da Silva and Sofia Santos Venceslau. 2011. A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and *archaea*, *Frontiers in Microbiology*, 2:69

M.C. Marques participated in the genomic analysis of hydrogenases and in table preparation.

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M.C. Marques participated in the writing and figure and table preparation.

1.1 HYDROGEN, A SUSTAINABLE ENERGY CARRIER

As global fossil fuel supplies drop and atmospheric carbon concentrations rise, the pressure is on to find viable alternatives to petroleum products. Hydrogen (H_2) produced from biological, renewable sources is a promising sustainable energy carrier as alternative to fossil fuels [1, 2]. Although hydrogen is the most abundant element in the Universe, the H_2 can only be found on Earth, in relatively small concentrations, close to the site where it is produced (geochemically or biologically), because it easily escapes from the earth's atmosphere. The hypothesis that the enzyme hydrogenase may have been among the first enzymes to evolve is reinforced by the fact that in the early Earth the atmosphere was reducing and rich in H_2 [3]. Hydrogen is mostly found chemically bound to other elements and H_2 is an energy rich molecule that releases over 100 kJ per gram when oxidized to water, and as such can act as a low potential reductant, $E^\ominus (H_2/H^+) (abs) = 4.40 \text{ V}$, for cellular energy generation and anabolic processes [4–6]. At present, 88% of commercial hydrogen derives from fossil reserves [6, 7]. This practice is an environmental paradox because this clean fuel is being generated from a polluting and limited source. Moreover, there are several limitations to the use of H_2 . Low density, high platinum prices and H_2 storage difficulty hinder the implementation of this green technology. However, H_2 production from biological organisms is an attractive strategy and may play a very important role in the development of a hydrogen-based economy [8–10].

1.2 BIOLOGICAL HYDROGEN PRODUCTION

H₂ production typically occurs in organisms occupying anoxic, low potential environments and is often used as a redox valve to release excess reductant [11, 12]. The synthesis of H₂ in these organisms allows the regeneration of oxidized electron carriers and the continuation of energy generation via fermentation. Organisms from all three kingdoms of life can utilize H₂ in their metabolism: anaerobes, facultative anaerobes, aerobes, photosynthetic bacteria, cyanobacteria and eukaryotes [2, 4, 13–16]., using metalloenzymes named hydrogenases (Hases). These enzymes utilize protons for the synthesis of H₂. Depending on the metal ion composition of the active site, Hases can be classified as nickel-iron (NiFe), iron-iron (FeFe) or iron-only (Fe) Hases [17–19].

Photobiological H₂ production by microalgae is particularly attractive as the main inputs for the process (water and solar energy) are abundant. Recent developments in solar-driven H₂ production in green algae have been achieved [20, 21], with particular emphasis on the model organism *Chlamydomonas reinhardtii* [22]. Biological H₂ production using fermentative bacteria also emerges as a promising alternative to fossil fuels [4, 6, 14, 23]. Extensive studies have been carried out to enhance H₂ production by pure bacteria and mixed cultures [14, 24]. Amongst the various biological processes to produce hydrogen, dark fermentation offers a tremendous potential for useful applications such as treatment of organic wastes [25].

Another route to produce renewable H₂ that appears very interesting is the development of solar water-splitting systems [26, 27]. This process relies on

highly efficient and stable catalysts for H₂ production, as natural enzymes that are able to reduce or oxidize water [28, 29] and that are better than artificial catalysts.

1.3 HYDROGEN UTILIZATION BY SRB

Sulphate reducing bacteria (SRB) are anaerobic prokaryotes found ubiquitously in nature [30, 31]. They employ a respiratory mechanism with sulphate as the terminal electron acceptor, giving rise to sulphide as the major metabolic end product. These organisms play an important role in global cycling of sulphur and carbon in anoxic environments, particularly in marine habitats due to the high sulphate concentration, where they are responsible for up to 50% of carbon remineralization [32]. Sulphate reduction can be physiologically linked to hydrogen oxidation. Great progress has been made in the knowledge of the pathways and the enzymes used in the degradation of substrates by sulphate reducers. Most biochemical studies have focused on mesophilic sulphate reducers of the *Deltaproteobacteria*, mostly *Desulfovibrio* spp. [30, 33], but previous analyses indicated that the composition of energy metabolism proteins can vary significantly between different SRB [33–35].

The increasing number of available SRB genomes from different classes of both Bacteria and Archaea, allowed in 2011 a comparative analysis of Hase gene distribution in 25 SRB genomes available at the Integrated Microbial Genomes website. These included 3 Archaea, 17 *Deltaproteobacteria* (of the *Desulfovibrionaceae*, *Desulfomicrobiaceae*, *Desulfobacteraceae*, *Desulfohalobiaceae*, *Desulfobulbaceae*, and *Syntrophobacteraceae* families), 4 *Clostridia* (of the *Peptococcaceae* and *Thermoanaerobacterales* families), and *T.*

yellowstonii DSM 11347 of the Nitrospira phylum. The Hases found in this survey are represented in Tables 1.1 and 1.2.

The total absence of Hases in two SRB (*Caldivirga maquilingensis* and *Desulfococcus oleavorans* Hxd3) was unexpected and indicates that hydrogen metabolism is not essential for sulphate reduction. An unexpectedly high number of soluble cytoplasmic Hases, of both NiFe and FeFe families, were detected. *Desulfovibrio* organisms in particular have long been known to possess multiple Hases [34, 36]. These enzymes work mainly in H₂ consumption, but may also function for H₂ production, forming a complete H₂ metabolic cycle. A model proposed by Odom and Peck, stated that, during growth on organic acids, H₂ is produced in the cytoplasm by a cytoplasmic Hase from the oxidation of lactate or pyruvate, which then diffuses across the cytoplasmic membrane into the periplasm, where H₂ oxidation takes place. The generated electrons are then again imported across the membrane for cytoplasmic reduction of sulphate, whereas the generated protons are imported through ATP synthase for ATP synthesis. [37]. However, recent results with *D. gigas* (*Dg*) indicated that H₂ cycling is not indispensable for growth [38]. Furthermore, it has been shown that sulphate-reducing bacteria (SRB) display an extremely high Hase activity and can produce hydrogen fermentatively, whenever sulphate is growth-limiting in their natural habitats [39]. Moreover, the production of H₂ is affected by the metal cofactors available since expression of Hases is strongly dependent on the metal availability [40].

Table 1.1 Analysis of periplasmic Hases distribution in SRB genomes adapted from [41].

			Periplasmic NiFe				Periplasmic FeFe	
	N _T	N _P	Soluble		Memb		Sol	Memb
			HynAB	HysAB	Hyn ABC	Hyn ABC ₃	HydAB	FeFe _{mem}
Archaea								
<i>Archaeoglobus fulgidus</i>	2	1			1			
<i>Archaeoglobus profundus</i>	2	1			1			
<i>Caldivirga maquilensis</i>								
Deltaproteobacteria								
Desulfovibrionaceae								
<i>Desulfovibrio aesopaeensis</i>	3	2	1				1	
<i>Desulfovibrio desulfuricans</i> G20	6	4	1	1		1	1	
<i>Desulfovibrio desulfuricans</i> ATCC 27774	5	3	1		1		1	
<i>Desulfovibrio magneticus</i> RS-1	8	3	2				1	
<i>Desulfovibrio piger</i>	4	2	1	1				
<i>Desulfovibrio salexigens</i>	5	3	1	1			1	
<i>Desulfovibrio</i> sp. FW1012B	5	2	1				1	
<i>Desulfovibrio vulgaris</i> Hildenborough	7	4	1	1		1	1	
Desulfomicrobiaceae								
<i>Desulfomicrobium baculatum</i>	2	2	1	1				
Desulfobacteraceae								
<i>Desulfatibacillum alkenivorans</i>	3	1	1					
<i>Desulfobacterium autotrophicum</i> HRM2	6	2	1	1				
<i>Desulfococcus oleovorans</i> Hxd3								
Desulfogallobiaceae								
<i>Desulfogallobium retbaense</i> DSM 5692	2	1	1					
<i>Desulfonatronospira thiodismutans</i> ASO3-1	3							

N_T, total number of Hases; N_P, number of periplasmic Hases; FeFe_{mem}, membrane-associated FeFe Hase; FeFe_{bif}, cytoplasmic NAD(P)-dependent Hases; FeFe_{mon}, monomeric Fd-dependent Hases.

Table 1.1 Analysis of periplasmic Hases distribution in SRB genomes adapted from [41].

			Periplasmic [NiFe]				Periplasmic FeFe	
	N _T	N _P	Soluble		Memb		Sol	Memb
			HynAB	HysAB	Hyn ABC	Hyn ABC ₃	HydAB	FeFe _{mem}
<i>Desulfobulbaceae</i>								
<i>Desulfotalea psychrophila</i>	6	2	1		1			
<i>Desulfurivibrio alkaliphilus</i>	4	2	1		1			
<i>Syntrophobacteraceae</i>								
<i>Syntrophobacter fumaroxidans</i> MPOB	9	2	1				1	
Clostridia								
<i>Peptococcaceae</i>								
<i>Desulfotomaculum acetoxidans</i> DSM 771	4	1						1
<i>Desulfotomaculum reducens</i>	7	1						1
<i>C. Desulforudis audaxviator</i> MP104C	7	1						1
<i>Thermoanaerobacterales</i>								
<i>Ammonifex degensii</i> KC4	5	2		1				1
Nitrospirae								
<i>Thermodesulfovibrio yellowstonii</i>	5	1		1				
No. of organisms			15	8	5	2	8	4

N_T, total number of Hases; N_p, number of periplasmic Hases; FeFe_{mem}, membrane-associated FeFe Hase; FeFe_{bif}, cytoplasmic NAD(P)-dependent Hases; FeFe_{mon}, monomeric Fd-dependent Hases.

Table 1.2 Analysis of cytoplasmic Hases distribution in SRB genomes adapted from [41].

			Cytoplasmic NiFe							Cytoplasmic FeFe			
	N _T	N _P	Soluble					Memb		Soluble			
			HdrA-Mvh	HdrABC-Mvh	Mvh	Hox	Sens.	Ech	Coo	[FeFe] _{bif}	[FeFe] _{mon}	FHL	HsfB
Archaea													
<i>Archaeoglobus fulgidus</i>	2	1		1									
<i>Archaeoglobus profundus</i>	2	1		1									
<i>Caldivirga maquilensis</i>													
Deltaproteobacteria													
Desulfovibrionaceae													
<i>Desulfovibrio aespoeensis</i>	3	2						1					
<i>Desulfovibrio desulfuricans</i> G20	6	4									1	1	
<i>Desulfovibrio desulfuricans</i> ATCC 27774	5	3						1	1				
<i>Desulfovibrio magneticus</i> RS-1	8	3						1		2		1	1
<i>Desulfovibrio piger</i>	4	2							1		1		
<i>Desulfovibrio salexigens</i>	5	3						1				1	
<i>Desulfovibrio</i> sp. FW1012B	5	2						1		1			1
<i>Desulfovibrio vulgaris</i> Hildenborough	7	4						1	1	1			
Desulfomicrobiaceae													
<i>Desulfomicrobium baculatum</i>	2	2											
Desulfobacteraceae													
<i>Desulfatibacillum alkenivorans</i>	3	1	1				1						
<i>Desulfobacterium autotrophicum</i> HRM2	6	2	1							1		1	1
<i>Desulfococcus oleovorans</i> Hxd3													
Desulfobalobiaceae													
<i>Desulfobalobium retbaense</i> DSM 5692	2	1	1										
<i>Desulfonatronospira thiodismutans</i> ASO3-1	3		1	1			1						

Table 1.2 Analysis of cytoplasmic Hases distribution in SRB genomes adapted from [41] (cont.).

			Cytoplasmic NiFe							Cytoplasmic FeFe			
	N _T	N _P	Soluble					Memb		Soluble			
			HdrA-Mvh	HdrABC-Mvh	Mvh	Hox	Sens.	Ech	Coo	[FeFe] _{bif}	[FeFe] _{mon}	FHL	HsfB
<i>Desulfobulbaceae</i>													
<i>Desulfotalea psychrophila</i>	6	2	1			1					1	1	
<i>Desulfurivibrio alkaliphilus</i>	4	2			1	1							
<i>Syntrophobacteraceae</i>													
<i>Syntrophobacter fumaroxidans</i> MPOB	9	2	2		1	1				1	1		1
Clostridia													
<i>Peptococcaceae</i>													
<i>Desulfotomaculum acetoxidans</i> DSM 771	4	1								1	1		1
<i>Desulfotomaculum reducens</i>	7	1								3	2		1
<i>C. Desulforudis audaxviator</i> MP104C	7	1			1			1		1	3		
<i>Thermoanaerobacterales</i>													
<i>Ammonifex degensii</i> KC4	5	2			1						2		
Nitrospirae													
<i>Thermodesulfovibrio yellowstonii</i>	5	1		1	1						2		
No. of organisms			6	4	5	3	2	7	3	8	9	5	6

N_T, total number of Hases; N_P, number of periplasmic Hases; FeFe_{mem}, membrane-associated FeFe Hase; FeFe_{bif}, cytoplasmic NAD(P)-dependent Hases; FeFe_{mon}, monomeric Fd-dependent Hases.

1.4 HYDROGENASES

Hases were first described by Stephenson and Stickland in 1931 [42] and catalyse the reversible conversion of hydrogen described by Equation 1:



Although the reaction appears to be simple, metalloenzymes that catalyse this reaction are extremely complex. Catalysis takes place at a specialized metal centre that is able to split the H_2 molecule heterolytically. In the case of the reverse reaction, heterogenesis of H_2 results from H^+/H^- coupling [17, 18, 43]. Although the reactions catalysed by Hases are reversible, the enzymes are often biased in one direction *in vivo* (H_2 oxidation or H^+ reduction), but under special conditions such as in the presence of an unfavourable electron donor or acceptor, they can also catalyse the reverse reaction [12, 17, 44].

As mentioned previously, Hases can be classified as nickel-iron (NiFe), iron-iron (FeFe) or iron-only (Fe) Hases [17–19]. Hases from these three classes differ in polypeptide chain sequence, composition and structure of the active site metal centre. Nevertheless, a common feature of the active site in NiFe and FeFe Hases is that small inorganic CN^- and CO moieties are ligands to the iron atom, giving rise to very specific signals in Fourier Transform Infrared (FTIR) Spectroscopy that can be used as redox probes of the active sites [45–47]. These ligands are believed to maintain the Fe in a low-spin state to facilitate the activation of hydrogen [48]. FTIR spectra sensitivity to the redox state of the Hase active site affords a powerful tool to examine the active site *in situ* [49].

Hases contain iron-sulphur (Fe-S) clusters, involved in the electron

transfer pathway between the active site and the molecular surface. These [FeS] clusters are called proximal, medial and distal, according to their spatial localization relative to the active site. Electron transfer proceeds via the proximal, medial and distal clusters directing the electrons to the physiological electron acceptor (or donor) of the Hase [18, 33]. Furthermore, several studies aimed to map the submolecular pathways between the active site and the enzyme surface channelling H₂, proton, oxygen and CO [50–52].

To date there is no evidence for a single proton transfer pathway; in alternative, several possible pathways have been proposed [48]. Possibly, a chain of water molecules and other groups act as proton transfer groups, but an accurate establishment of any such pathway in each class of enzymes needs to be confirmed by mutagenesis studies. Since Xe atoms are similar in size to H₂ molecules, in 1997 Montet and co-workers [50] used diffusion of Xenon gas under pressure in *Dg* Hase crystals to investigate H₂ diffusion pathways into the active site. These authors concluded that, rather than randomly diffusing through the protein, the hydrogen molecule travels through specific hydrophobic channels, highlighted by ten Xe-binding sites located from difference Fourier electron density maps [50].

NiFe Hases are the most common group and the first crystal structure of a NiFe Hase (from the *Dg* enzyme) was reported in 1995 by Volbeda *et al.* at 2.85 Å resolution [47, 53]. FeFe Hases are generally monomeric [18, 54, 55] and their metal centre is formed by two Fe atoms. Many *Clostridia* species, as well as other bacteria, have been shown by genomic analysis to contain putative multimeric hydrogenases [54, 56]. FeFe Hases are phylogenetically unrelated to NiFe Hases [15, 17]. Since NiFe and FeFe Hases share no sequence similarity, they are an example of convergent evolution since they display common

structural features at the active site, as described above. FeFe Hases are often involved in H₂ production, whereas NiFe Hases appear to be more responsible for H₂ uptake [18, 57]. This physiological role seems to be related to their cellular localization: mostly cytoplasmic for the FeFe Hases and mostly periplasmic for the NiFe Hases [17, 19], despite some exceptions [41]. Hases found in the periplasm or cytoplasm, can be membrane-bound or soluble, although in eukaryotic cells they are frequently found in specialized compartments [15]. In contrast to NiFe Hases, FeFe Hases are generally more active in production of H₂. Turnover frequencies in the order of 10,000 s⁻¹ have been reported in the literature for FeFe Hase from *Clostridium pasteurianum* (Cp) [58]. Nevertheless, this family is very sensitive to O₂ making them less useful for biotechnological applications.

The Fe Hases are found in only a few species of methanogenic *Archaea* and their expression is induced under nickel-limited conditions [59]. They were initially referred to as "metal free" Hases, but were later renamed iron-sulphur cluster-free Hases or Fe-Hases as their active site contains a monomeric Fe [16, 59]. These rather unique enzymes catalyse the reversible reduction of methenyltetrahydromethanopterin with H₂ to methylenetetrahydromethanopterin and a proton. The reduction of the methenyl-substrate with H₂ is an intermediary step in the biological conversion of carbon dioxide to methane [15, 16, 18].

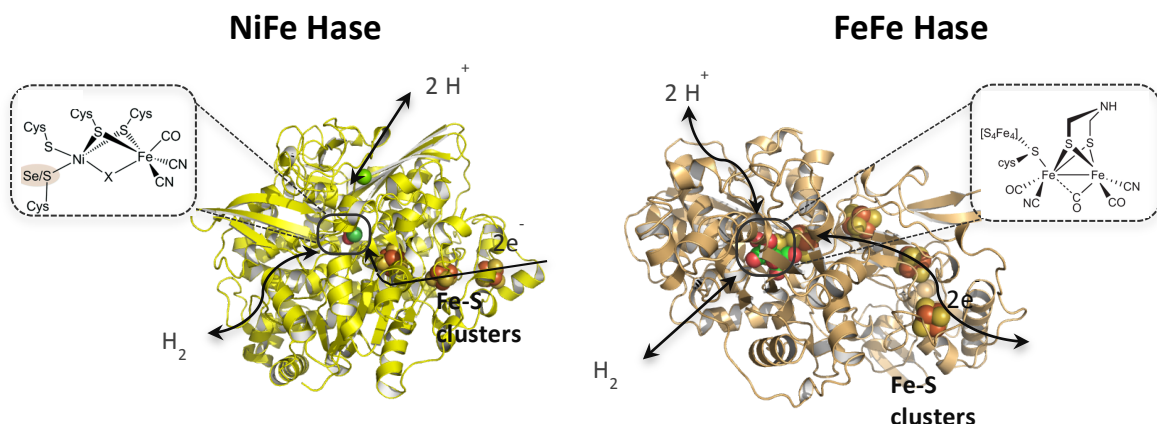


Figure 1.1. Ribbon diagrams of the structures of the heterodimeric NiFe Hase from *Dg* [53] and the monomeric FeFe Hase from *Clostridium pasteurianum* (*Cp*) [60]. Coordinates are from the Protein Data bank files 1yq9 and 1feh, respectively. Atoms in the active site and FeS clusters are represented as spheres and coloured orange for iron, yellow for sulphur, green for carbon, red for oxygen, blue for nitrogen, bright orange for nickel and dark green for chloride. Possible proton pathways and hydrogen diffusion channels are schematically represented.

1.5 NIFE HYDROGENASES

The NiFe Hases comprise at least two subunits: the catalytic subunit that contains the active site, and the electron transferring subunit that contains one or more Fe-S clusters. The binuclear active site includes one Fe atom and one Ni atom, attached to the protein chain by four cysteine residues. Two of these bridge the two metals whereas the other two only bind Ni. The Fe atom is in turn coordinated by three small diatomic ligands, two CN^- and one CO. In some NiFe Hases one of the Ni-binding cysteines is a Selenocysteine (Sec), and these constitute the NiFeSe subgroup (see section 1.6).

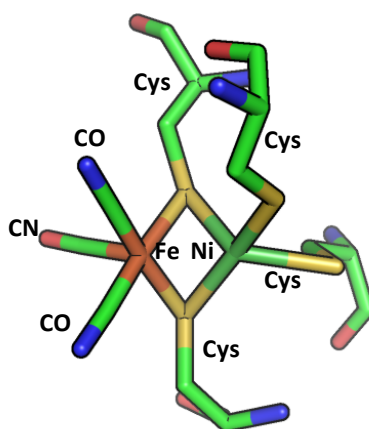


Figure 1.2 Schematic view of the Ni-Fe active site.

Phylogenetic analysis of the two core subunits of NiFe Hases reveals that these proteins can be subdivided into four subgroups, which present different quaternary structures, cellular locations and physiological functions [15, 16] (Table 1.3). Group 1 includes the periplasmic H_2 -uptake Hases, which oxidize H_2 and in most cases reduce the quinone pool.

Table 1.3 Classification of NiFe Hases, adapted from [61]

Group	Subgroup	Function
1	-	Periplasmic H ₂ -uptake Hases
2	a	Cyanobacterial uptake Hases
	b	H ₂ -sensing Hases
3	a	F ₄₂₀ -reducing Hases
	b	Bifunctional NAD(P) Hases
	c	F ₄₂₀ -nonreducing or methyl viologen reducing Hases
	d	Bidirectional NAD(P)-linked Hases
4		Membrane-bound H ₂ evolving Hases

H₂ oxidation in anaerobic respiration is linked to the reduction of electron acceptors such as NO₃⁻, SO₄²⁻, fumarate or CO₂. Periplasmic H₂-uptake Hases are characterized by the presence of a signal peptide in the small subunit, involved in translocation of the two mature subunits to the periplasm [62, 63]. Most group 1 Hases are associated with a third subunit, a cytochrome *b* responsible for membrane anchoring and quinone reduction, with the exception of periplasmic Hases of *Desulfovibrio* spp. and other sulphate reducers, where their redox partner is a soluble low-potential tetraheme cytochrome *c*₃ that transfers electrons to membrane-associated complexes [33].

Group 2 Hases are cytoplasmic and include the cyanobacterial uptake enzymes [64] as well as the H₂-sensors that regulate expression of other Hases [65]. The group 3 Hases are cytoplasmic enzymes with additional subunits, which bind soluble cofactors such as NAD(P) and cofactor F₄₂₀, but include also the heterodisulphide reductase-associated Hases (previously known as methyl-

viologen reducing Hases or F420-non-reducing Hases). This group includes many enzymes from methanogens [66], but some are also present in bacteria. They are called bidirectional because they may function reversibly in physiological conditions. Group 4 is formed by energy-conserving membrane-bound Hases, which are multimeric protein complexes with several transmembrane subunits [15, 67]. These proteins are involved in energy conservation and their subunits are closely related to subunits of complex I [68–70].

The most studied so-called “standard” NiFe (including NiFeSe) Hases belong to group 1 and many of the classical examples were isolated from *Desulfovibrio* species. Additionally, whereas most Hases are inactivated by O₂, a few so-called “oxygen-tolerant” Hases have been described to be able to oxidize H₂ in the presence of O₂ at near atmospheric levels. These group 1 O₂-tolerant NiFe Hases are found in *Escherichia coli* (*E. coli*) and *Ralstonia eutropha* H16 (*Re*), among other aerobic or facultative aerobic H₂-oxidizing bacteria [71], and have also been the object of intense studies in recent years [18, 71]. These Hases are not really tolerant to O₂, since they also form inactive species, but they recover quickly from O₂ inactivation.

1.5.1 NiFe Hases maturation

The knowledge of the maturation pathway of NiFe Hases is fundamental for the design of heterologous expression systems for these enzymes. It has been shown that the maturation of Hases is a remarkably complex process [72–74]. Most of the information gathered for this process was obtained from NiFe Hases of *E. coli*, *Re*, *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* and

Azotobacter species [75].

NiFe Hases are enzymes with complex metal clusters whose biosynthesis demands the coordination of three basic processes, namely the formation of the apo-protein, the uptake and provision of the metal(s) and the assembly of the centre to generate the catalytically active protein [57, 73–76]. From all the three basic processes, it is recognized that the metal uptake and centre assembly are the steps less characterized. Furthermore, the rates of metal uptake, metal availability, accumulation and its specific donation to the maturation machinery are considered fundamental processes [75].

Nickel incorporation into the metal centre requires a set of accessory proteins [57, 74, 75], of which the HypAB complex is known to play a role [18]. At present, it is known that for a large number of organisms, several genes exist coding for maturation and regulatory proteins downstream of the structural genes. The accessory genes can also be rearranged in several transcription units. For *DvH*, some selected ones are listed in Table 1.4. Maturation includes enzymes for the synthesis of the CN and CO ligands as well as mechanisms for their addition to the active site iron. Moreover, because the metal centre is hidden inside the heterodimer, a mechanism ensuring that the apo-protein is accessible to the incoming metal is also necessary. A further conformational event certifies that it is buried inside the holoenzyme after insertion is completed [75].

Table 1.4 NiFe Hase operon with all the structural and maturation genes known for DvH, deduced from the genome sequence. All the genes from the organism with the same first word e.g. *hyp* belong to the same operon. Adapted from [77].

Function	DvH genes
Small subunit	<i>hynA</i>
Large subunit	<i>hynB</i>
C-terminal peptidase	<i>hynC</i>
Ni-incorporation/maturation	<i>hypA</i>
Ni-insertion	<i>hypB</i>
Chaperone maturation	<i>hynD</i>
Ni-incorporation/maturation	<i>hypD</i>
Purine derivative binding	<i>hypE</i>
CN/CO delivery	<i>hypF</i>

The presence of such genes in DvH has been reported from the genome sequence project [78], using sequence homology. A succinct explanation of the roles of the various genes is introduced here and described in Figure 1.3 (for the Ni-Fe cofactor assembly), following the nomenclature as shown for DvH:

❖ HynD/ HypC: chaperone/maturation

In the first step of maturation, HypC forms a complex with the precursor of the large subunit, helped by conserved cysteines, to hold it in a proper conformation, which makes it accessible for metal insertion [72].

❖ HypD: CN/CO delivery

HypD is a monomeric protein that forms a complex with HypC in the maturation cycle [72].

❖ HypA/HypB: Ni insertion and Ni incorporation/maturation

HypA is a cysteine-rich protein with four conserved cysteines, arranged into two CxxC motifs. They are proposed to be redox proteins carrying a Fe-S cluster. HypA cooperates with HypB in the insertion of nickel into the precursor of the large hydrogenase subunit [74]. HypB has a conserved gene sequence, and especially the presence of His residues, indicating a divalent metal binding function. These proteins were found to bind and store nickel and have a conserved GTP-binding motif at the carboxyl terminus, and shown to be essential for nickel insertion [57, 74, 75].

❖ HypE: Purine derivative binding, and CN/CO delivery

HypE together with HypF catalyses the synthesis of the CN ligands of the active site iron of the NiFe Hases using carbamoylphosphate as a substrate [72].

❖ HypF: CN/CO delivery

The active site assembly starts with the insertion of the three diatomic Fe ligands. HypF plays a crucial role in this process together with HypE, although HypD might be also involved [72, 79].

❖ HynC: endopeptidase

As the last step of maturation, a C-terminal segment of the Hase large subunit is cleaved off by an endopeptidase, after the conserved sequence motif His/Arg/Gln [75, 76].

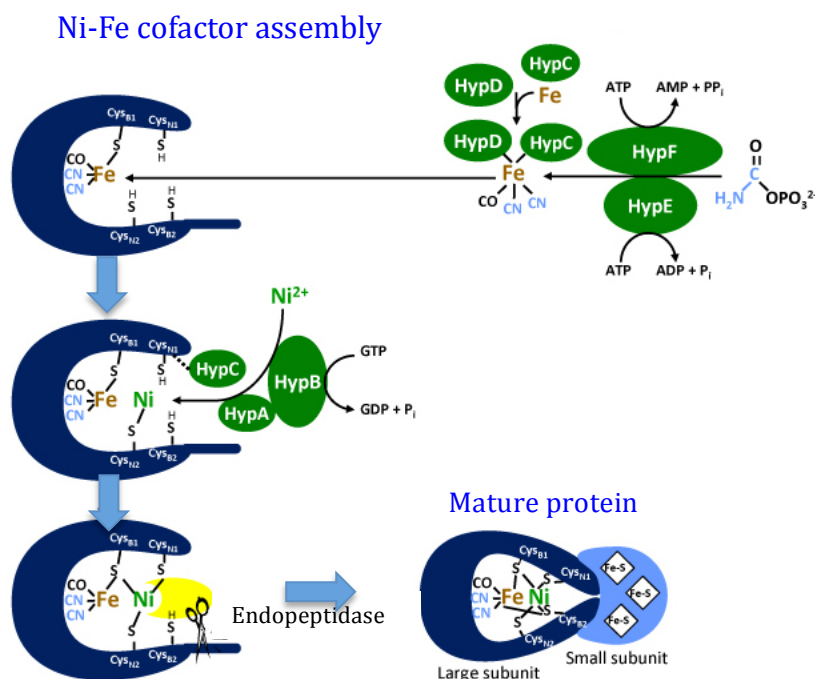


Figure 1.3 Current accepted model for the Ni-Fe cofactor assembly. Adapted from [73].

The current model for the NiFe centre and [Fe-S] assembly relies on information integrated from several biological systems. Nevertheless, the knowledge is still too scarce to fully comprehend the maturation process of NiFe Hases and more studies are needed. For example, it is still unknown if there are specific proteins for the maturation of NiFeSe Hases, although given the small difference between them, this would not be expected.

1.5.2 Tat Protein Export Pathway

The twin-arginine translocation pathway (Tat pathway) is a protein export, or secretion pathway found in plants, bacteria, and archaea [80]. In contrast to the Sec pathway, which transports unfolded proteins, the Tat

pathway serves to actively translocate folded proteins across a lipid membrane bilayer. In bacteria, the Tat translocase is found in the cytoplasmic membrane and serves to export proteins to the cell envelope, or to the extracellular space [81] .

NiFe Hases are known to be translocated by the Tat pathway [18]. As a consequence, all the NiFe Hase maturation proteins operate in the cytoplasm. The [FeS] cluster assembly and NiFe active site incorporation occurs in the cytoplasm and after the process is finished the catalytic active Hases are translocated across the cytoplasmic membrane by the Tat pathway [81]. This is true for periplasmic-facing NiFe Hases even if they are bound to the membrane. The characteristic signal peptide termed as twin-arginine motif is located in the N-terminal of the NiFe Hases small-subunit and is cleaved by a signal peptidase, after the fully matured protein is transported to the periplasm [57]. Depending on being a membrane-bound or a periplasmic soluble Hase, it will finally bind the cytochrome b or interact with the cytochrome c-type electron acceptor, respectively.

1.5.3 The catalytic cycle of NiFe Hases

Based on spectroscopic and electrochemical data, a number of intermediates in the catalytic cycle of NiFe Hases have been assigned [18, 49, 82, 83]. Nickel is the redox-active metal ion responsible for the heterolytic splitting of dihydrogen. A new redox state is produced whether an electron is accepted or donated during the course of the reaction. All these redox states are characterized as active or inactive, depending on whether the enzyme is ready to catalyse the reaction or not.

Standard NiFe Hases – even those from anaerobic organisms – are usually purified under aerobic conditions, and the purified enzymes are obtained in a mixture of oxidized states, named Ni-A and Ni-B [84]. These inactive states of the enzyme are proposed to contain a bridging oxo-group (likely a peroxide or hydroperoxide molecule) between the nickel and iron atoms, although the exact identity is yet the subject of discussion [18, 49, 83, 85]. Catalytic activity can be (partially) regenerated by incubating the oxidized enzyme in reducing conditions under an atmosphere of H_2 . The Ni-A state is designated as unready-inactive and the Ni-B as ready-inactive, because the Ni-A state requires much longer incubation times to obtain an active enzyme than the Ni-B state, which can be quickly reduced to a catalytically active form [47]. The catalytic states of the NiFe active site were initially characterized by EPR spectroscopy, but this only provides insight into the paramagnetic states of the NiFe cluster. More recently, use of FTIR spectroscopy allowed the analysis of all redox states of the NiFe site, as represented in Table 1.5 for *Av* and *Dg* NiFe Hases. Only slight differences in frequency values, redox potentials (Figure 1.4) and pH-dependence have been observed among different enzymes. This implies that the structure and functionality of the NiFe active site in the majority of the NiFe Hases are comparable [47].

The catalytic cycle of the NiFe site has been elucidated with a reasonable degree of accuracy (Figure 1.4). The inactive oxidized states Ni-A and Ni-B pass through Ni-SU and Ni-SI_r, respectively before entering the catalytic cycle (states Ni-SI_a, Ni-C and Ni-R), described in Figure 1.4.

Table 1.5 Vibrational frequencies of the Fourier transform IR bands of *Allochromatium vinosum* (Av) and *Dg* NiFe Hases at different redox states, adapted from [86, 87].

Redox State	Infra-red bands (cm ⁻¹)			
	<i>Allochromatium vinosum</i>		<i>Desulfovibrio gigas</i>	
	CO	CN	CO	CN
Ni-A	1945	2083, 2093	1947	2083, 2093
Ni-B	1944	2079, 2090	1946	2079, 2090
Ni-C	1950	2074, 2087	1952	2073, 2086
Ni-L	1898	2043, 2058	-	-
Ni-R	1936	2059, 2073	1940	2060, 2073

Ni-S states are EPR silent. The fully reduced and EPR silent state, Ni-R, is reached after two consecutive electron reductions of the Ni-SI_a state. These results suggest a reaction mechanism that keeps the Fe in a low-spin and low oxidation state throughout all reaction intermediates, whereas the Ni switches oxidation states between a diamagnetic Ni (II) and a paramagnetic Ni (III) [44]. Besides the redox states represented in Figure 1.3, other paramagnetic states can be formed, namely Ni-L, when Ni-C is illuminated at low temperatures, and CO-inhibited states, that are generated from Ni-SI_a or Ni-L [18, 49]. Studies of the redox states in “non standard” NiFe Hases have also been reported, for NiFeSe Hases, O₂-tolerant Hases and hyperthermophilic Hases [88–92]. In the

O₂-tolerant Hases, only the easily reactivated Ni-B state is observed after O₂ exposure; these enzymes do not display the unready oxidized Ni-A state, [71], which explains their fast reactivation profile. On the other hand, in the NiFeSe Hases neither Ni-A nor Ni-B states are formed after O₂ exposure.

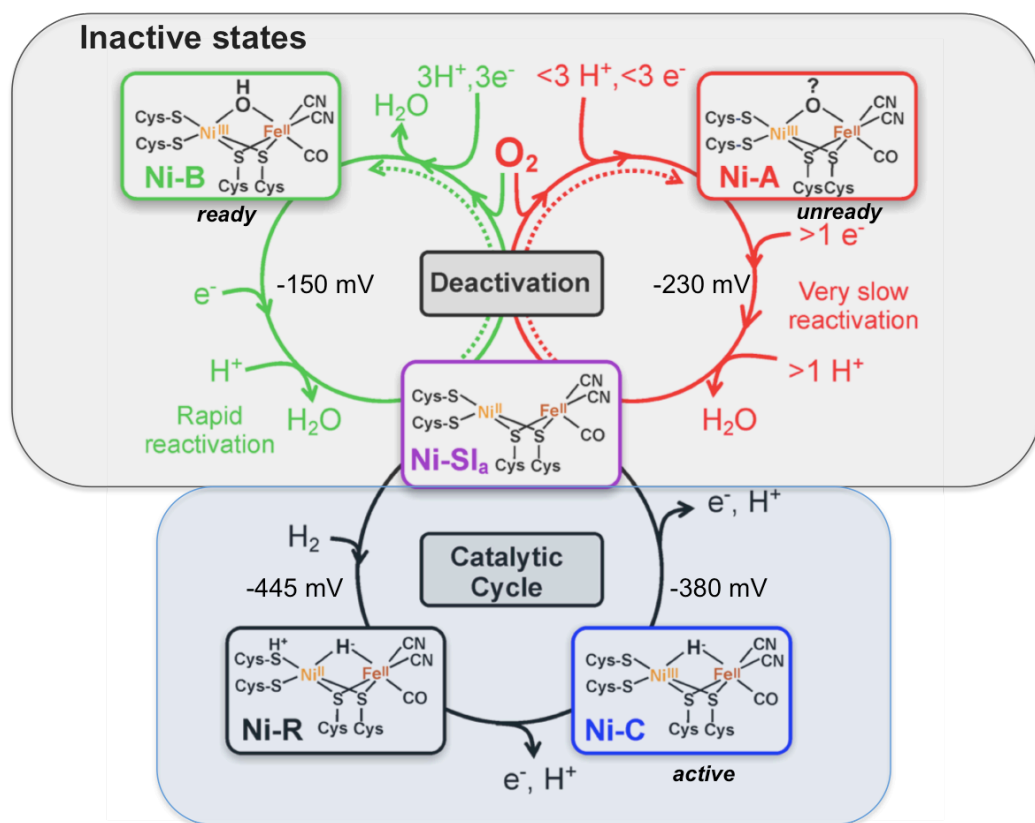


Figure 1.4 Scheme showing the currently proposed catalytic cycle and inactivation/reactivation mechanism for NiFe Hases, adapted from [18]. Aerobic inactivation is indicated by solid arrows (red and green). The formal redox potentials (at pH 8.0) correspond to those measured by FTIR-spectroelectrochemistry of *Dg* Hase [49]. All states denoted by “S” are EPR-silent. In the Ni-A state the identity of the bridging ligand X is still controversial. As recently reported [85] the Ni-A state can also be reached under anaerobic conditions.

1.5.4 O₂-tolerant NiFe Hases

Certain microorganisms, including pathogens, can utilize H₂ during aerobic growth [17]. These organisms possess NiFe Hases that can exhibit H₂ oxidation activity even in the presence O₂ [90, 93, 94].

Structures of these O₂-tolerant Hases, particularly from *Re* and *Hydrogenovibrio marinus* (*Hm*), were recently determined and revealed the presence of a novel [4Fe-3S] cluster proximal to the active site [95, 96]. Conventional [4Fe-4S] centres are usually coordinated by four Cys residues (see Chapter III). In contrast, the proximal cluster in these Hases is ligated by six Cys residues, and the sulphurs of the two extra Cys residues substitute one of the four inorganic sulphides, leading to an open and flexible 6Cys-[4Fe-3S] architecture [71, 93]. This unprecedented cluster is proposed to not only participate in electron transfer to the catalytic centre, but also to donate two electrons and one proton for the reduction of O₂, thus avoiding the formation of the unready, inactive Ni-A state of the enzyme [18, 71, 96]. It is well-known that the word “ready” and the Ni-B state are linked, and the Ni-B state has been established as a Ni (III) species with a OH⁻ ligand in the bridging position [18, 47]. A recent model for O₂ tolerance of these enzymes emphasized that, upon O₂ attack O₂-tolerant Hases must immediately supply all four electrons needed to produce one H₂O molecule [71], thereby preventing the formation of reactive oxygen species (ROS) that could damage or block the active site [90], leading to the Ni-A state found in standard NiFe Hases.

The importance of the additional Cys ligands in the coordination of the proximal cluster was assessed by Goris and his co-workers [97]. The substitution of the two additional Cys residues, in *Re* MBH, by two glycines had a strong

impact on the electronic properties of the proximal Fe-S cluster, namely the disappearance of a paramagnetic species present at high redox potential (absent in standard NiFe Hases), and converted the enzyme into a O₂-sensitive Hase, as shown by protein film electrochemistry experiments [71, 97].

The extensive study of O₂-tolerant enzymes *Re* MBH, *E. coli* Hydrogenase-1 (*Ec* Hyd-1) and *Aquifex aeolicus* I (*Aa* Hase I) yielded a list of characteristics shared by these enzymes:

- ❖ No formation of the Ni-A state following exposure of the enzyme to O₂;
- ❖ Resistance to CO, a strong inhibitor of NiFe Hases;
- ❖ Catalytic bias toward H₂ oxidation versus proton reduction;
- ❖ Fast activation of Ni-B under mildly reducing conditions at a given potential, known as E_{switch};
- ❖ Unusual EPR spectrum under oxidizing conditions, indicating the presence of at least one additional paramagnetic centre at high potential;
- ❖ Two conserved cysteines as additional ligands to the proximal Fe-S cluster, which correspond to conserved glycines in standard NiFe Hases.

Recently, Pandelia and coworkers evaluated the phylogeny of Group 1 NiFe Hases, paying specific attention to proximal [FeS] cluster as structural marker characteristic of Hases complexes. This study led to the conclusion that the most successful evolutionary invention of O₂-tolerant NiFe enzymes is this six Cys coordination in the proximal [FeS] cluster [98]. It is proposed that the expression of this special cluster is linked to H₂ oxidation, when O₂ is present and is the terminal electron acceptor of these organisms [99]. In fact we can observe in Figure 1.5, that the six Cys proximal [FeS] has the highest redox potential of all [FeS] described [100].

Remarkably, some microorganisms possess NiFeSe Hases, where a Sec residue is terminally coordinating the Ni instead of a Cys. This alteration appears to afford some level of protection to O₂, as these enzymes also never form the inactive Ni-A state [61] and sustain partial H₂ production under low O₂ concentrations [101].

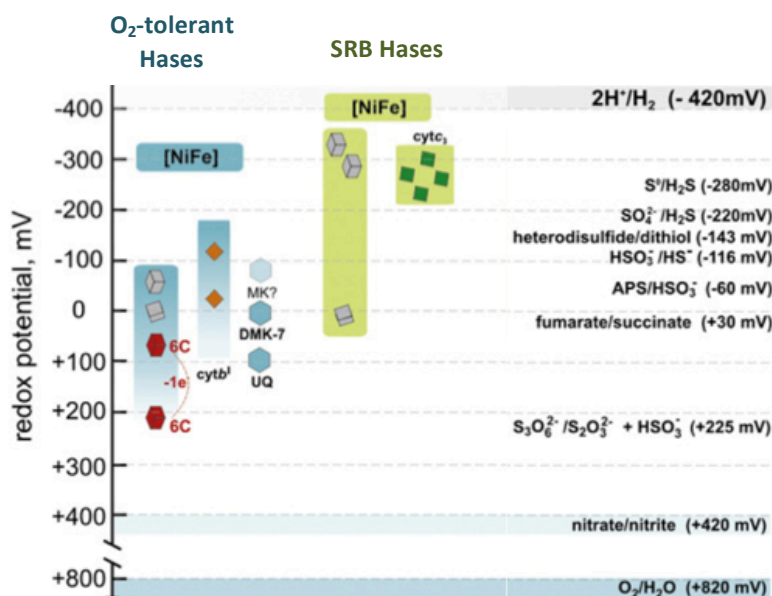


Figure 1.5 Redox scheme of the different Group 1 enzymes constructed from spectroscopic and biochemical data, adapted from [99]. O₂-tolerant Hases: the NiFe, Fe-S clusters and cyt *b* redox potentials are those measured for *Aa* Hase I. SRB-Hases: redox potentials shown are for *Dv*MF and *Dg*. On the right are listed the redox potentials of different electron acceptors. Note that sulphate is a much weaker oxidant than O₂

1.6 NiFeSe Hases

Se is a trace element essential for different organisms, including humans. It is fundamental for the correct function of the immune system as well for cellular defence against oxidative damage [102–104]. It is incorporated into proteins in the form of the amino-acid selenocysteine (Sec or U), which is structurally similar to cysteine although more reactive [105–107]. In selenocysteine, a selenol group replaces the thiol group. Insertion of Sec into a protein is a much more complex process than for any of the other 20 amino acids. This is because its codon (UGA) is the same as the stop codon, so there has to be a special machinery to recode it as a sense codon for Sec [105, 108]. Sec usage is found in the three domains of life, suggesting its presence in the last universal common ancestor [109–111]. A genomic analysis revealed that about 25% of bacteria with sequenced genomes utilize Sec and have from 1 to 39 selenoproteins, with the most selenoprotein-rich organisms belonging to Deltaproteobacteria and Clostridia [112]. Despite the high energetic cost of selenoprotein biosynthesis there is a low evolutionary exchange rate between Sec and Cys [113–115]. The most common event is Cys-to-Sec replacement, which is balanced in evolutionary terms with loss of selenoprotein genes [113, 116]. Lateral gene transfer events also play a role, but neither the loss nor the acquisition of Sec-usage is irreversible. Thus, most selenoproteins have evolved from a Cys-containing ancestor.

It is not yet fully understood why organisms contain this unusual amino acid; a plausible explanation is that Sec usage might be related to its physicochemical properties, which is why it is almost exclusively found in the catalytic site of several redox-active enzymes. The use of Sec may be partly

justified by its high nucleophilicity and the fact that the selenol group can be active at lower pH [105]. Organoselenium compounds are frequently more reactive, in particular in the case of oxidation-reduction reactions. Examples of selenoenzymes are glutathione peroxidase, thioredoxin reductase, formate dehydrogenases and NiFeSe Hases [108, 117–119]. The first two proteins are involved in the oxidative stress metabolism. Because Sec presence is common in proteins associated with oxidative stress responses, some authors claim that, as a highly oxidizable amino acid, Sec could only be kept in aerobic systems that developed specific protective mechanisms to deal with the possible oxidative damage of the Sec residues [120, 121]. Another point of view regarding Sec-enzymes considers them as a highly sophisticated innovation that leads to a catalytic advantage, as Sec-containing enzymes are usually orders of magnitude more active than their Cys-containing counterparts [107, 122]: this is observed also in NiFeSe Hases vs. standard NiFe Hases. In *Desulfovibrio* spp. the periplasmic H₂-uptake NiFeSe Hases (Hys Hases) are particularly interesting because in solution assays with artificial mediators they display higher activities for H₂ production than for H₂ oxidation, which is the reverse of what is observed for their Cys-homologues (Table 1.6).

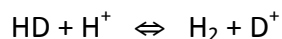
Higher H₂ production to H₂ oxidation ratios in NiFeSe enzymes relative to standard NiFe Hases, have also been reported from electrochemical studies [127]. Two additional important properties were described for the NiFeSe Hase from *Db*: it shows less inhibition by H₂ than standard NiFe Hases, and it can sustain H₂ production in the presence of small quantities of O₂ [128], making this enzyme a very attractive catalyst for biological hydrogen production.

Table 1.6 Catalytic activities of Hases (in U.mg⁻¹). Adapted from [61]

Group 1 Hases	H ₂ production	H ₂ uptake
<i>DvH</i> HysAB ^[a] (Sec)	6908	900
<i>Db</i> HysAB ^[b] (Sec)	2000	-
<i>D. sallexigens</i> HysAB ^[c] (Sec)	1830	1300
<i>Db</i> New Jersey HysAB ^[d] (Sec)	8600	-
<i>Dg</i> HynAB ^[e] (Cys)	440	1500

[a] From ref.[123] [b] From ref [124] [c] From ref.[125] [d] From ref.[126]

Another measure of Hase activity is by their catalysis of H₂ isotope exchange in D₂O or D₂ in H₂O. For example, in the D₂/H₂O medium assay the following reactions take place:



The activity is monitored with a mass spectrometer by measuring masses 2 (H₂), 3 (HD) and 4 (D₂). The experimental observation that HD is detected as an intermediate of the reaction proves that dihydrogen oxidation occurs via heterolytic cleavage to a proton and a hydride. A characteristic property of the NiFeSe (Hys) Hases is that they catalyse the double isotope exchange of D₂ with protons faster than the single one, contrary to standard NiFe (Hyn) Hases [125, 127]. This distinct catalytic behaviour is attributed to the lower pK_a of the active site Sec (5.2) compared to that of the equivalent cysteine (8.0) in standard NiFe Hases [129]. This terminal ligand of the Ni atom is believed to be the base that accepts the proton produced during the heterolytic cleavage of dihydrogen at the active site. In consequence, the higher acidity of Sec vs. Cys should lead to a faster exchange of protons, thus favouring the double exchange product

formation during the isotope-exchange assay, as well as the H₂-production activity.

A common feature of the NiFeSe Hases is the presence of three [4Fe4S]^{2+/1+} clusters in the small subunit, rather than a mesial [3Fe4S]^{1+/0} observed in the standard NiFe Hases, such as in the *Dg* enzyme. As mentioned, these clusters are responsible for electron transfer with the redox partner. The involvement of a [3Fe4S]^{1+/0} cluster is unexpected due to its high redox potential, but it may not prevent fast electron tunnelling due to the proximity of the [4Fe4S]^{2+/1+} clusters [130]. To address this question a mutant of the *D. fructosovorans* (*Df*) NiFe Hase was constructed with a [4Fe4S]^{2+/1+} instead of a [3Fe4S]^{1+/0} cluster [131]. This resulted in a mesial cluster with a 300 mV lower redox potential, but there was no significant effect in terms of the catalytic activity. This contrasts with the results observed in a mutant of the *M. voltae* group 3 cytoplasmic Hase containing a [3Fe4S]^{1+/0} instead of a [4Fe4S]^{2+/1+} cluster, which reduced F₄₂₀ with a ten-fold lower rate [132], indicating that in this enzyme the low redox potential of the [4Fe4S]^{2+/1+} cluster is important for efficient electron transfer with its redox partner.

1.6.1 Spectroscopic studies

The UV-visible spectra of NiFeSe Hases show the expected absorption band at approximately 400 nm due to the iron-sulphur clusters [133, 134]. Many EPR studies of NiFeSe Hases have been done since the mid-eighties in order to detect these iron-sulphur clusters and the Ni atom in their paramagnetic states [134]. These studies revealed that Sec is a direct ligand to the active site Ni [132, 134, 135], and showed aerobically oxidized NiFeSe Hases to be EPR-silent, an indication that these enzymes have neither the

[3Fe4S]^{1+/0} cluster present in most H₂-uptake NiFe Hases nor do they form the inactive Ni (III) states [132, 134, 135]. Mössbauer and EPR evidence suggested that only two [4Fe4S]^{2+/1+} clusters were present in the *Db* enzyme [136]. The crystallographic structures of the NiFeSe Hases from *Db* [137] and *DvH* [138] later confirmed the Sec coordination to Ni and the absence of a [3Fe4S]^{1+/0} cluster, showing that three [4Fe4S]^{2+/1+} clusters are present in the small subunit. The absence of Ni(III) signals in the EPR spectra of the oxidized NiFeSe enzymes is also an important difference relative to NiFe Hases. It has been proposed that the absence of the Ni-A and Ni-B paramagnetic signals, typical of standard NiFe Hases in their inactive oxidized states, is correlated with the fast activation upon reduction of oxidized NiFeSe Hases [133, 139]. Upon partial reduction of these enzymes with H₂, a rhombic EPR signal can be assigned to a Ni(III) state that is reminiscent of the Ni-C state of NiFe Hases, in which a hydride bridges both active site metals [140]. There are few differences between the NiFeSe Hases isolated from different deltaproteobacterial microorganisms, suggesting that the structure of their bimetallic centre in the reduced active state is nearly identical. Redox titration of the Ni-C state has been performed for the *Db* Hase. The formal redox potential measured was -300 mV at pH 8.0 vs SHE [133]. EPR studies with ⁷⁷Se-enriched Hases from *Db* [124] and *M. voltae* F₄₂₀-nonreducing Hase [141] and group 3 cytoplasmic Hase [142] showed hyperfine splitting of the Ni-C signal due to magnetic interaction with the spin of the ⁷⁷Se nucleus (I = ½), indicating the existence of a Se-Ni bond and a partial delocalization to the Se atom of the unpaired electronic spin of Ni(III). These results demonstrated that Sec is coordinated to the Ni site before any NiFeSe Hase crystallographic structure became available. In contrast, hyperfine splitting of the Ni-C signal due to the ¹H nucleus is considerably smaller, leading only to a slight

broadening of the signal in H₂O relative to D₂O [143, 144]. Müller *et al.* compared the distribution of protons and unpaired electrons in the active site of the *Db* NiFeSe Hase with the *Dg* NiFe Hase by high-resolution ¹H electron nuclear double resonance (ENDOR) spectroscopy. The authors concluded in this work that only two proton ENDOR signals were different between the two Hases, which were assigned to the β-methylene hydrogen atoms of the terminal Cys ligand of the Ni, which is replaced by a Sec in *Db* Hase [144]. The results therefore suggested that, besides the change of a S atom by a Se atom, the structure of the bimetallic Ni-Fe centre in its active state is very similar in both types of Hases. This was later confirmed by the crystal structures. Upon illumination of Hases in the Ni-C state at temperatures around 30 K, a new EPR signal appears that has been named Ni-L and has been assigned to a Ni¹⁺ state in which a proton has been photodissociated from the active site, presumably originating from the bridging hydride ligand [143, 145].

The EPR spectra of reduced NiFeSe Hases at temperatures lower than 20 K yield signals arising from the [4Fe4S]^{2+/1+} clusters, which are fast relaxing species that are not visible at higher temperatures [123, 133, 134, 139]. Interaction of the spin of the proximal [4Fe4S]^{2+/1+} cluster with the spin of Ni(III) leads to a splitting of the Ni-C signal below 20 K.

FTIR spectroscopy has also proved to be a very powerful tool for characterization of the active site of NiFe(Se) and FeFe Hases. Wang *et al.* reported the first two FTIR spectra of a NiFeSe Hase, from the as-isolated and H₂-reduced enzyme forms of the *Db* enzyme [146]. Both reported spectra are quite complex because a large number of CO and CN⁻ peaks were present, suggesting sample heterogeneity and making signal assignment difficult. A complete characterization by FTIR spectroscopy of the NiFeSe Hase from *DvH* in

its different redox states has been reported by De Lacey *et al.* [147]. Two conformations of the active site were detected by FTIR for each redox state, and each conformation had a set of one CO band and two CN⁻ bands as shown in Table 1.6. Two different EPR-silent, air-stable redox states that are not in equilibrium were also identified. One of these states corresponds to the as isolated enzyme upon aerobic purification (Ni-IS), whereas the other corresponds to reoxidized enzyme after reduction with H₂ or other reductants (Ni-OX). Upon reduction of these states the catalytically active states Ni-C, the paramagnetic state detected by EPR, and Ni-R, a completely reduced EPR-silent state, immediately appear.

Table 1.7 Vibrational frequencies of the Fourier transform Infra-Red (IR) bands of DvH NiFeSe Hase at different redox states, adapted from [147].

Redox State	Wavenumber, cm ⁻¹			
	Isoform I		Isoform II	
	CO	CN	CO	CN
Ni-IS	1904	2076, 2085	1939	2079, 2094
Ni-OX	1938	2084, 2095	1944	2074, 2102
Ni-TR	1925	2078, 2092	-	-
Ni-C	1915	2083, 2094	1900	2068, -
Ni-R	1933	2064, 2079	1909	2064, 2079

A transient state (Ni-TR) was detected in some experiments when Ni-OX was reduced to Ni-C. The active states Ni-C and Ni-R are in redox equilibrium, and a formal redox potential of -380 mV was measured at pH 8.0 [147]. Figure

1.6 shows a summary of the redox steps between the different active site states of the NiFeSe Hase from *DvH*, as deduced from the FTIR results. Binding of the CO inhibitor to the active site of this Hase has also been studied by FTIR spectroscopy. The results showed that the extrinsic CO binds to Ni in both conformations of the active site in a similar way to standard NiFe Hases, although in one of the CO-inhibited conformations the active site of the NiFeSe Hase is more protected against attack by O₂ [127]. This is in agreement with the Ni-CO EPR signal detected, which also suggested direct binding of extrinsic CO to Ni, although in this case the FTIR and the EPR spectra do not correspond to the same Ni redox state. The former probably corresponds to a Ni(II)-CO state [127], whereas the latter was assigned to a Ni(I)-CO state [142]. Spectroscopic studies of NiFeSe Hases by other techniques other than EPR and FTIR have been reported [119, 148, 149], but these are beyond the scope of this work and will not be discussed here.

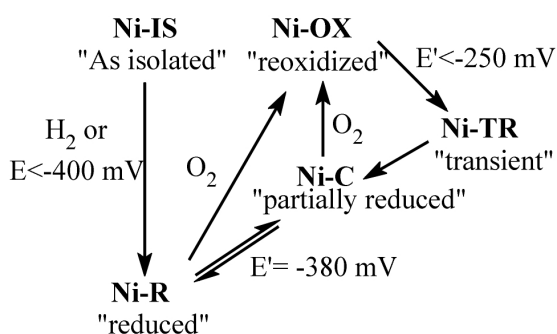


Figure 1.6 Scheme of the different redox states of *DvH* NiFeSe Hase, adapted from [147].

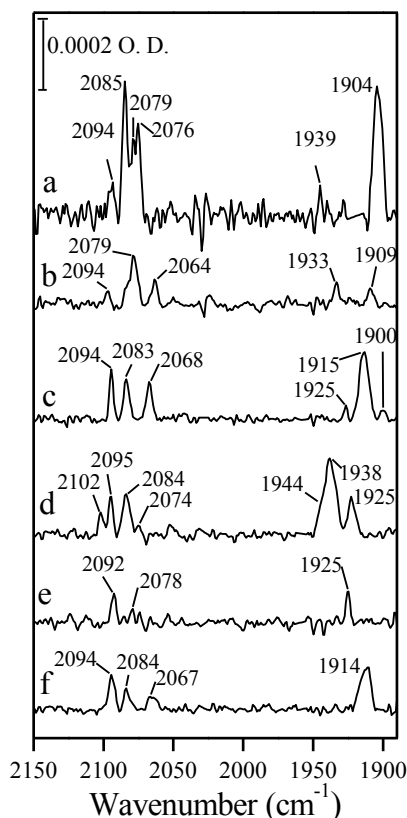


Figure 1.7 FTIR spectra of 1.0 mM NiFeSe Hase from *DvH* poised in a spectroelectrochemical cell under different redox conditions. a) as isolated, open circuit potential was +300 mV (Ni-IS); b) reduced at an applied potential of -435 mV (Ni-R); c) reoxidized at -250 mV (Ni-C); d) left overnight at open circuit (Ni-OX); e) reduced for 10 min at -300 mV (Ni-TR); f) reduced for 20 min at -300 mV. Adapted from [147].

1.6.2 Structural studies

The first X-ray crystallographic structure of a NiFeSe Hase, the periplasmic enzyme from *Db* in a fully reduced active form, was published in 1999 at 2.15 Å resolution [137]. This structure confirmed that Sec is a ligand to the Ni and

revealed that the oxo bridging ligand hitherto observed in oxidized NiFe Hases is absent in the reduced state. It also showed that the small subunit included three $[4\text{Fe}4\text{S}]^{2+/1+}$ clusters, and not two as predicted spectroscopically, with the $[3\text{Fe}4\text{S}]^{1+/0}$ mesial cluster of standard NiFe Hases (see below) replaced by a $[4\text{Fe}4\text{S}]^{2+/1+}$ cluster. The second three-dimensional structure of a Sec-containing Hase, that of the oxidized soluble form of NiFeSe Hase from *DvH*, was published 12 years later [138]. This was the first structure of an "as isolated" oxidized Hase from the NiFe family where a bridging oxide ligand is not present at the NiFe active site, providing definitive evidence that the oxidized species of the Sec-containing Hases are distinct from those of standard NiFe Hases.

Prior to the start of this thesis work these were the only crystal structures available of NiFeSe Hases. In both cases the domain structure of the large and small subunits is typical of group 1 uptake NiFe Hases [33, 137, 138, 150] (Figure 1.8). The coordination of the active site and of the $[4\text{Fe}4\text{S}]^{2+/1+}$ clusters is identical to that described in the NiFe Hases from SRB, with the notable difference that the mesial iron–sulphur cluster is an $[4\text{Fe}4\text{S}]^{2+/1+}$ centre, as opposed to the $[3\text{Fe}4\text{S}]^{1+/0}$ cluster of the NiFe Hases [46, 53]. The presence of three $[4\text{Fe}4\text{S}]^{2+/1+}$ centres seems to be a conserved feature in NiFeSe Hases, including those from methanogens. In the *DvH* NiFeSe Hase structure, an extrinsic sulphur atom bound to the Ni atom was found at the active site with partial occupation. As a result, the Sec ligand to the Ni is present in three conformations (see Figure 1.9), as interpreted from the electron density: in two of them, Sec binds the extrinsic sulphur whereas in the third conformer the sulphur is absent.

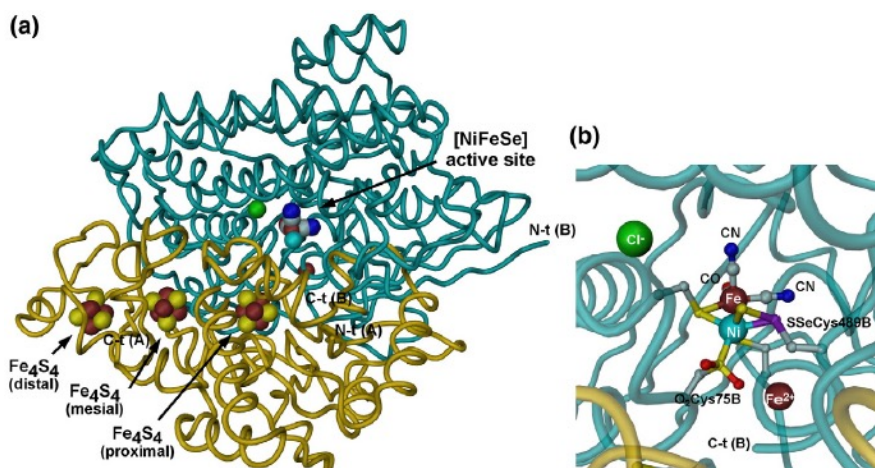


Figure 1.8. The DvH NiFeSe Hase structure. (a) C α tube diagram representation of the overall structure, with cofactors represented as spheres; the small subunit is coloured in gold and the large subunit is coloured in dark cyan. (b) Detail view of the active site. For clarity, only the conformer I of Sec489B is represented. Atom colours are light blue for carbon, red for oxygen, blue for nitrogen, yellow for sulphur, brown for iron, cyan for nickel and green for Cl⁻. Adapted from [138]

Conformer I is likely to correspond to the conformation of the oxidized state, whereas conformer III is identical to the Sec conformer of the fully reduced state in the *Db* Hase structure [137]. Conformer II may be an intermediate between the other two conformers. The oxidized state of the protein therefore includes an extrinsic sulphur atom bound to the Se and Ni, resulting in a Sec conformation that is distinct from the reduced state [138]. Interestingly, the known structures of oxidized and reduced NiFe Hases show the equivalent Cys residue always in the same conformation, regardless of the oxidation state of the enzyme.

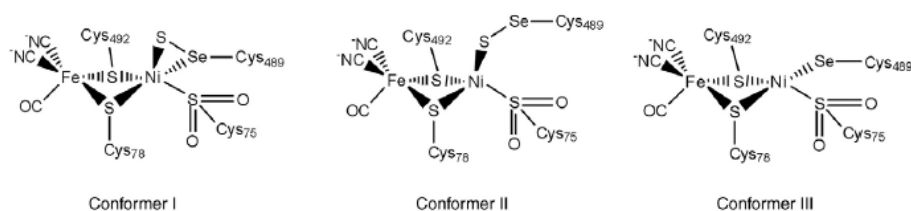


Figure 1.9 Schematic representation of the three Sec 489 conformers in the large subunit of *DvH* NiFeSe Hase.

Several high-resolution reports of NiFe Hases crystal structures from *DvMF* and *Df* [19, 151] mentioned the oxidation of an active site cysteine, generally involving a single oxygen atom (sulphenate) at either one of the bridging or terminal cysteines. Surprisingly, in *DvH* Hase the terminal cysteine is present in a doubly oxidized state (sulphinate), whereas still bonded to the Ni atom (2.27 Å). This feature has not been reported before in other Hases. The proximal iron–sulphur cluster of the *DvH* NiFeSe Hase in the as-isolated state, was also found to be partially oxidized, in a presumed [4Fe-3S-3O] form [138] (see Chapter 3), a modification which had previously been observed in the three-dimensional structure of the NiFe Hase from *D. desulfuricans* ATCC 27774 (*Dd*) [152].

In addition to the NiFe active site and the iron–sulphur clusters, the structures of the NiFeSe Hases from *Db* and *DvH* contain a $\text{Fe}^{2+/3+}$ ion octahedrally coordinated by three water molecules, a main-chain carbonyl oxygen, a glutamic acid carboxylate oxygen and a nitrogen atom from the imidazole ring of the C-terminal histidine in the large subunit [61, 109]. This histidine residue is highly conserved among several NiFe Hases [17] and the Fe coordination environment is identical to that of a Mg^{2+} ion found at the equivalent site in NiFe Hase structures [151–153]. A further ligand species is

present in the crystal structures of *Db* and *DvH*: a H_2S molecule and a Cl^- ion, respectively, located in similar positions and *ca.* 7 Å distant from the active site [137, 138]. This site may represent a storage location for the extrinsic sulphur atom (as H_2S) when the enzyme is in its reduced state. An interesting consequence of the lack of a NiFe bridging ligand in the known structures of NiFeSe Hases is that the Ni-Fe distance remains practically constant (2.5 Å) between oxidized and reduced forms of the enzyme. This contrasts with NiFe Hases, where the removal of the oxide bridging ligand upon enzyme reduction leads to the shortening of the Ni-Fe distance (e.g., from 2.8 Å to 2.6 Å in NiFe Hase from *DvMF*) [121].

The structure of *DvH* NiFeSe Hase provides important evidence regarding the oxygen tolerance of NiFeSe Hases, which allows them to produce H_2 even in the presence of low levels of O_2 [128]. The mechanism of O_2 tolerance of Hases has been probed by several studies [154], namely by measuring H_2 oxidation activity in the presence of O_2 over a range of potentials, pHs and temperatures. Replacement of Cys by Sec in the active site is the most obvious difference between the Se-containing and the standard NiFe Hases. In the *DvH* NiFeSe Hase the Sec conformations in conformers I and II have the effect of shielding the Ni atom in the active site, blocking its access by other molecules such as O_2 . Only in a reduced-like conformation does the side chain conformation of Sec expose the Ni atom [138].

Both NiFeSe Hases with known structure, as well as almost all NiFe Hases structurally characterized to date, belong to group 1, with the exception of the recent first X-ray structure of a Group 3 NiFe Hase [155]. Therefore, there is no experimental structural information about the NiFeSe Hases belonging to the other groups, namely groups 3a and 3b.

1.6.1 O₂ tolerance and applications

Research on Hases aims at the development of biotechnological applications, either by their direct use in fuel cells or biosensors, by engineering organisms to increase their H₂-productivity via biophotolysis or fermentation, or by the development of biomimetic synthetic catalysts for H₂ production [156, 157]. One of the main problems associated with the applications of Hases is their reactivity towards oxygen, which leads to introduction of oxygen species in the active site and enzyme inactivation [19, 158]. In this respect, the NiFe Hases have a more favourable profile because their inactivation is reversible, whereas the reaction of FeFe Hases with O₂ leads to irreversible damage [49, 156, 159, 160].

A few NiFe Hases isolated from aerobic hydrogen-oxidizing bacteria display a very high tolerance to O₂ [161]. However, these NiFe Hases have a low activity of H₂ production, due to product inhibition [154]. The NiFeSe Hases, in contrast, display high activities in H₂ production and oxidation, and were initially believed to be oxygen-tolerant because they do not display either the long reactivation step observed for typical NiFe Hases after exposure to oxygen [133], or the characteristic inactivated Ni-A and Ni-B species after aerobic isolation [123, 145, 161, 162]. However, it was recently shown that the periplasmic enzymes from *Db* and *DvH* are indeed inactivated by oxygen, but display a very fast reactivation step, like the O₂-tolerant Hases, but requiring a lower redox potential [127, 128]. The crystal structure of the oxidized as-isolated *DvH* Hase showed that no oxide bridging ligand is present at the Ni-Fe site, in contrast with standard NiFe Hases, but a terminal Cys was found oxidized to sulphinate [138].

NiFeSe Hases are interesting targets for biotechnological applications. In addition to their quick reactivation after O₂ exposure, Sec-containing Hases are particularly attractive catalysts due to their high activities, in particular of H₂ production, which was shown to be tolerant to low concentrations of O₂, as well as to display low levels of H₂ inhibition [128]. These properties were explored by adsorbing the *Db* Hase enzyme to TiO₂ nanoparticles together with a ruthenium photosensitizer, which enabled photocatalytic H₂ production [29], or even more recently in a dye-sensitized TiO₂ with soluble Eosin Y (organic dye) which allowed the production of H₂ under high levels of O₂ [26]. In another application the *DvH* enzyme was bound to a gold electrode allowing for direct electron transfer, and moderate electrocatalytic currents for H₂ production were observed [127].

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CHAPTER 2

Influence of the protein structure surrounding the active site on the catalytic activity of NiFeSe Hydrogenases

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Marta C. Marques has purified the NiFeSe Hase necessary for the experiments and participated in the preliminary experimental work, during a short stay in the Instituto de Catálisis e Petroleoquímica, CSIC, Madrid.

ABSTRACT

A combined experimental and theoretical study of the catalytic activity of a NiFeSe Hase has been performed by H/D exchange mass-spectrometry and molecular dynamics (MD) simulations. Hases are enzymes that catalyse the heterolytic cleavage or production of H_2 . The NiFeSe Hases belong to a subgroup of the NiFe enzymes in which a selenocysteine is a ligand of the Ni atom in the active site instead of cysteine. The aim of this research is to determine how much are the specific catalytic properties of this Hase influenced by the replacement of a S by Se in the coordination of the bimetallic active site *versus* the changes in the protein structure surrounding the active site. The pH dependence of the D_2/H^+ exchange activity, together with the high isotope effect observed in the Michaelis constant for the dihydrogen substrate and in the single exchange/double exchange ratio suggests that a “cage effect” due to the protein structure surrounding the active site is modulating the enzymatic catalysis. This “cage effect” is supported by MD simulations of the diffusion of H_2 and D_2 from the outside to the inside of the protein, which show different accumulation of these substrates in a cavity next to the active site.

Keywords Hase · selenocysteine · enzyme kinetics · isotope exchange · molecular dynamics

2.1 INTRODUCTION

Hases have emerged as attractive enzymes for biotechnological applications [1, 2], but most importantly as source of inspiration for the development of biomimetic catalysts for H_2 production or for its oxidation in fuel cells [3-5]. Hases can catalyse this reaction in a reversible way with high turnover, under mild conditions and with almost no overpotential [1, 6]. The active site of Hases contains Ni and/or Fe as the only metals [7], thus nature shows us that efficient catalysis of H_2 production and oxidation is possible without using noble metals. In most NiFe Hases the nickel atom is coordinated by the thiol groups of four cysteine amino acids, two of them in a terminal position and the other two forming bridges with the Fe atom of the active site [7]. However, in some NiFe Hases one of the cysteines is replaced by a selenocysteine, as shown in Figure 2.1A. The interesting aspect of these NiFeSe Hases is that, although the chemical properties of Se and S are similar, the change of coordination of one cysteine by a selenocysteine does modify considerably the catalytic and spectroscopic features of the active site [8]. Some of the most notable catalytic properties of NiFeSe Hases are: a) they become active immediately upon reduction, whereas most NiFe Hases isolated under aerobic conditions require a slow reductive activation process; b) they have higher H_2 -production activity than H_2 -oxidation activity, contrary to most NiFe Hases; c) they are more tolerant towards oxygen in the H_2 -production activity than FeFe Hases and most NiFe Hases [8-12]. However, it is not yet clear if these differences are only due to the replacement of S by Se in the coordination sphere of the Ni or if the protein structure surrounding the active site also plays an important role [13, 14].

The catalytic activity of Hases involves several steps, which are all reversible: *a*) diffusion of hydrogen molecules to the active site through the protein, *b*) heterolytic cleavage of H_2 after binding to the bimetallic active site, leading to formation of an hydride and a proton, *c*) intra-molecular electron transfer from the active site to the protein surface, *iv*) proton transfer from the active site to the water solvent and *v*) inter-molecular electron transfer to the redox partner [6]. Crystallographic studies and molecular dynamics simulations point to the existence of hydrophobic channels and cavities that connect the active site of NiFe Hases with the molecule surface, through which H_2 will diffuse preferentially [15-17]. In a different direction from the gas channels, a chain of [FeS] clusters, separated by distances less than 1.5 nm, provides a fast electron-transfer pathway from the H_2 -splitting site to the site on the surface where electron acceptors bind [7]. A third pathway between the surface and the active site is required for transfer of protons involved in the Hase reaction. Several pathways have been proposed for NiFe Hases involving proton-exchanging groups of amino acids, such as aspartates, glutamates and histidines, and water molecules within the protein structure [7, 14, 18, 19]. There is general agreement in considering one of the terminal cysteine ligands bound to the Ni (the selenocysteine in NiFeSe Hases) as the first proton-accepting site after the heterolytic cleavage of H_2 [6, 7].

The catalytic activity of Hases can be characterized not only by H_2 -oxidation or H_2 -production assays, but also by the proton isotopes exchange reaction followed by mass spectrometry [9, 20, 21]. This kinetic assay shows the reversibility of Hases' catalytic activity, and is an intrinsic measurement of it because it is performed in the absence of a redox partner (protein, compound or electrode), which drives the kinetics of the reaction in one of the directions.

This assay is also used to test inorganic catalysts for hydrogenation reactions [22-24]. The experimental observation that HD is detected as an intermediate of the exchange reaction catalysed by Hases proves that dihydrogen oxidation occurs via heterolytic cleavage to a proton and a hydride, as shown in Figure 2.1B [20, 21]. A characteristic property of the NiFeSe Hases is that they catalyse the double isotope exchange of D_2 with protons faster than the single one, contrary to standard NiFe Hases [9, 20, 21, 25].

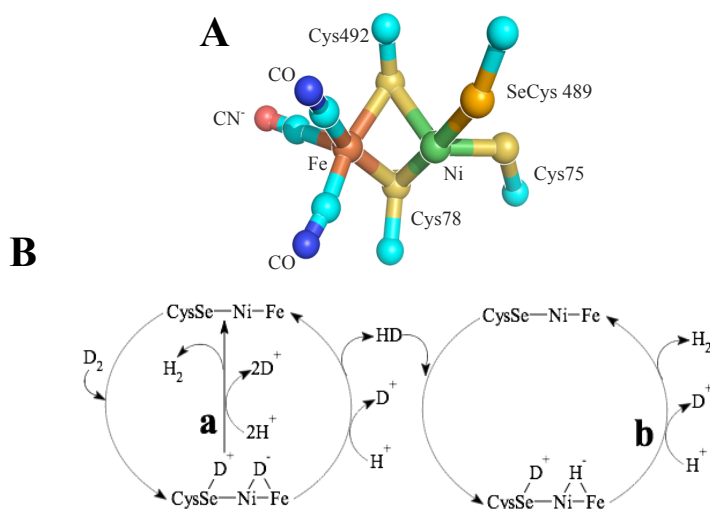


Figure 2.1 **A)** Structure of the active site of NiFeSe Hases. **B)** Catalytic mechanism of D_2/H^+ exchange by NiFeSe Hases. The high initial rate of the double exchange reaction can be explained by fast exchange at both the protic and hydride sites before dihydrogen release (a) or by successive reactions of the formed HD intermediate before being released out of the enzyme (b).

In this work we describe a joint experimental and theoretical study of the catalytic function of the NiFeSe Hase from *DvH* by H/D exchange mass-spectrometry and molecular dynamics (MD) simulations. This Hase is associated to the cytoplasmic membrane of the organism and displays the interesting catalytic properties of NiFeSe Hases [26, 27]. Moreover, its crystal structure has

been recently obtained by X-ray diffraction studies [28]. The aim of the study is to determine how much the specific catalytic properties of this enzyme are due to the replacement of a S by Se in the coordination of the bimetallic active site or to the differences in the protein structure surrounding the active site. The results of this study are not only interesting for understanding of the catalytic mechanism of Hases, but also for the design of biomimetic catalysts for hydrogenation reactions.

2.2 MATERIALS AND METHODS

2.2.1 Hase purification and preparation

DvH NiFeSe Hase was isolated and purified using purification protocols adapted from Valente et al. [26]. In the last step of purification a Pharmacia Q-Sepharose HP column (HiLoad 16/10) was used, which equilibrated with 20 mM Tris-HCl and 0.1% n-Dodecyl β -D-maltoside (DDM, Sigma). This column yielded pure the *DvH* NiFeSe Hase, confirmed by the SDS-PAGE and activity-stained native gel.

The concentration of the Hase sample for the activity assays was 0.1 μ M in 50 mM Tris pH 7.6, containing 0.1% DDM.

2.2.2 Hydrogen/deuterium exchange activity

The catalytic activity of *DvH* NiFeSe Hase was measured by membrane-inlet mass-spectrometry, in which a thermostated 37°C anaerobic vessel with magnetic stirring was connected through a 14 μ m Teflon membrane to a mass

spectrometer (Pfeiffer Prisma) [29]. The output signal of the spectrometer for each mass value is proportional to the partial pressure of the corresponding gas in the reaction vessel [11]. The output signal was first calibrated by saturating the reactor solution with 100% H₂ and then by 20% D₂ in 80% Ar (Air Liquide).

The D₂/H⁺ assays were done in 10 ml of buffered H₂O solutions in the pH range 3-10, previously purged with Ar (Air Liquide), and with initial concentrations of D₂ that ranged 0.4-100 μM. First, the solution in the reactor was bubbled with D₂ until the desired value of mass 4 signal was reached. After reaching this value, the reactor lid was closed so that no gas phase was left in the reactor. The enzymatic reaction was started by adding in succession 100 μL of 1 % DDM, 1 μL of 1 M sodium dithionite, to eliminate residual oxygen, and 25 μL of 0.1 μM Hase through a rubber septum with gas-tight syringes (Alltech). During the experiment, masses 2, 3 and 4 were scanned at 1 atomic mass unit/s. The initial rates of H₂ and HD production were determined from the difference in the slopes after and before Hase addition and were expressed in μmol.min⁻¹.mg protein⁻¹. The data correspond to mean values determined from at least 3 measurements.

For the H₂/D⁺ assays, the reactor contained 10 ml of 20 mM phosphate buffer in D₂O (99.9%, Sigma-Aldrich), pD 7.0 with initial H₂ concentrations that ranged between 7 and 450 μM. All other measurement parameters were the same as in the D₂/H⁺ assays. Previously, 1 μL of 100 μM Hase sample was incubated overnight in 999 μL of D₂O buffered solution at 4°C, in order to exchange the enzyme's protons to deuterons. A control activity assay was performed in the D₂/D₂O system, which showed that H₂ and HD production catalysed by the Hase, due to the presence of residual H⁺ in the deuterated solution, were negligible.

2.2.3 Molecular dynamics (MD) simulations

The diffusion of H₂ and D₂ from the outside to the inside of the NiFeSe Hase from *DvH* was simulated using MD simulations. We assumed that the redox states of the active site as described by Niu et al. for NiFe Hases [30] can be transposed to NiFeSe ones, as we have done before in our studies of the NiFeSe Hase from *Db* [14]. We choose to simulate the NiFeSe Hase from *DvH* in the Ni-SIIa redox state, which is the most oxidized of the active states of NiFe Hases active site [6]. All the [FeS] clusters of the Hase were considered to be in the oxidized state.

The program GROMACS version 4.0.4 [31] was used to run the MD simulations, with the GROMOS 54a7 force field [32]. The partial charges of the NiFeSe active site and the [FeS] clusters were calculated with quantum chemistry calculations, as described [14]. The van der Waals parameters of the Ni, Fe and Se were adapted from the Universal Force Field [33]. For H₂ we used the model described by Hunter et al. [34]. For D₂ we used the same model, but we doubled the mass of the two atoms. The simulations were started from the X-ray structure of *DvH* NiFeSe Hase determined by Marques et al. [28] (PDB ID 2WPN), with the protonation states at pH 7.0 determined by Poisson-Boltzmann calculations and Monte Carlo methods, as described previously [16]. The protein was solvated in a dodecahedral box using the SPC water molecules [35], to which we added 100 H₂ or 100 D₂ molecules. Ten replicates were simulated water for 50 ns. The equations of motion were integrated using a time step of 2 fs. We used the Berendsen thermostat and barostat to keep the temperature and pressure at 300 K and 1 atm respectively [36]. The number of H₂ or D₂ molecules added corresponds to a much higher concentration than the

normal solubility of this substance in water. However, the presence of such a high concentration is a necessity for obtaining reliable statistics and does not affect the integrity of the protein, as we verified by comparing the RMSD with respect to the crystal structure before and after the addition of the H_2 and D_2 molecules.

2.3 RESULTS

2.3.1 Hydrogen/deuterium exchange activity

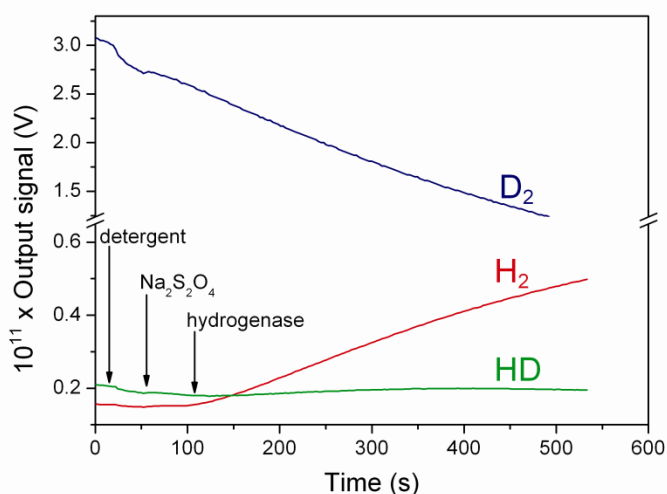


Figure 2.2 D_2/H^+ exchange activity of *DvH* NiFeSe Hase followed by mass spectrometry. Measurement done at 37°C in 20 mM phosphate buffer in H_2O , pH 7.0 and saturated with 20% D_2 , 80% N_2 . The arrows indicate additions of 0.01% DDM, 0.1 mM sodium dithionite and 0.25 nM Hase.

A representative measurement of the D_2/H^+ exchange activity of *DvH* NiFeSe Hase at pH 7.0 is shown in Figure 2.2. After hermetic closing of the reactor (time zero) the mass 4 signal starts to decrease due to the consumption

of the D_2 in the solution as it is sucked through the Teflon membrane by the high vacuum of the connected mass spectrometer. Only after the Hase was added did the signals of masses 2 and 3 start to increase due to H_2 and HD production. The previous addition of detergent to the reactor is necessary to retain the maximal catalytic activity of the membrane Hase, during the D_2/H^+ exchange activity assays [26, 37].

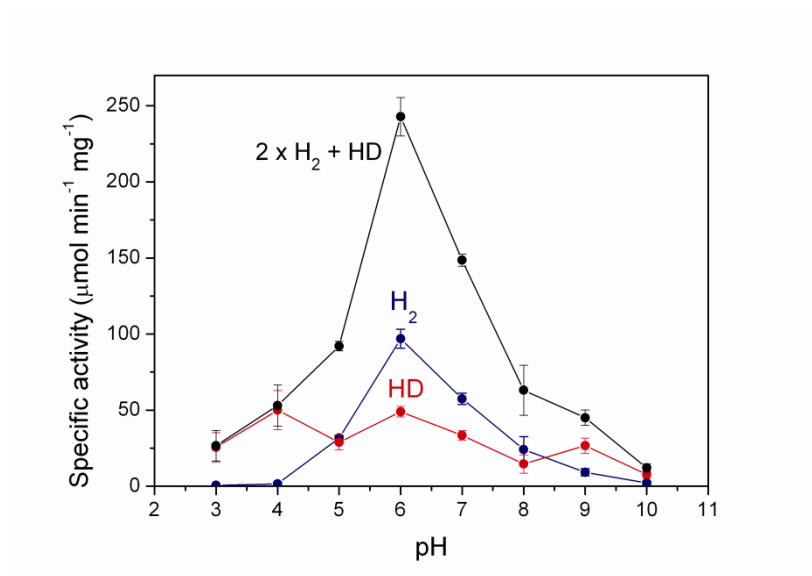


Figure 2.3 Dependence of the D_2/H^+ isotope exchange activity of *DvH* NiFeSe Hase on pH, and comparison of the rate double exchange production (H_2) with the single exchange production (HD). Measurements were done at 37°C with the following mixture of buffers: sodium citrate, MES, HEPES, TRIS and sodium carbonate, each of them at 10 mM concentration.

As expected, the isotope exchange activity measurements were less reproducible and gave lower rates of product formation when no detergent was added (not shown). Additionally, the presence of sodium dithionite as chemical reducer was compulsory in order to activate the Hase, although this process

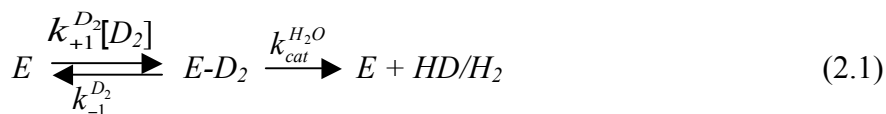
only required a few seconds before maximal production of H_2 and HD took place. As shown in Figure 2.2, it is clear that the rate of the double exchange product formation (H_2) is much higher than that of the single exchange product (HD), which is the typical kinetic behaviour of the NiFeSe Hases under such conditions [9, 11, 20, 25].

However, the D_2/H^+ isotope exchange activity was observed to be very dependent on the pH as shown in Figure 2.3. In this graph the trends of the specific activities of H_2 and HD production are represented. In addition, the trend of the overall D_2/H^+ exchange specific activity is represented as $2 \times \mu\text{mol } H_2 + \mu\text{mol HD}$ produced per minute because two protons are exchanged in the first case. The optimum of the overall isotope exchange activity is at pH 6, as well as for the H_2 production. Interestingly, the HD production activity is not so dependent on the pH and predominated over H_2 production at high and low pH values. In consequence, the H_2/HD ratio of the isotope exchange activity is only high around the pH optimum, decreasing greatly at the more acidic and basic pH values. Therefore, the double exchange reaction only occurs faster than the single exchange under the conditions in which the enzyme is working at the higher turnover values.

The dependence of the D_2/H^+ exchange activity of the NiFeSe Hase on the substrate concentration was also evaluated. The membrane-inlet mass-spectrometry method has the advantage of allowing the simultaneous measurement of products evolution rates (mass 2 and mass 3 signals) and the substrate concentration (proportional to mass 4) during the activity assay. Thus, the dependence of the specific activity on the concentration of substrate may be estimated from one single experiment in which the D_2 dissolved in the reactor cell is gradually consumed by the catalytic action of the Hase. However,

for correct interpretation of the kinetic results, the background uptake of the measured gasses by the spectrometer has to be considered. This background uptake was shown to be proportional to each gas concentration, although its correction requires complex kinetic equations and will increase experimental error at high concentrations of the measured gas [38]. Because of this, we have measured only the initial rates of HD and H₂ production for each experiment, because under these conditions the background uptake of these gasses by the spectrometer is very small. Furthermore, it can be easily eliminated by subtracting the slopes of mass 2 and mass 3 evolutions before enzyme addition from the ones after. Besides, the initial concentration of D₂ in the enzymatic reaction can be controlled by the measurement of the mass 4 signal at the moment of Hase injection.

The dependence of the *DvH* NiFeSe Hase D₂/H⁺ exchange activity on substrate concentration was evaluated by the Michaelis-Menten model [39] of enzyme kinetics:



$k_{+1}^{D_2}$ and $k_{-1}^{D_2}$ are the rates constants for the binding of the D₂ substrate to the enzyme (*E*) and its dissociation respectively. $k_{cat}^{H_2O}$ is the apparent first order rate constant of the catalytic conversion of the bound substrate to the products HD and H₂ at infinite concentration of D₂, and includes the following steps: a) heterolytic cleavage of D₂; b) exchange of one or two D⁺ by H⁺ from the solution; c) HD or H₂ formation; d) release of the product to the solution via the

Hase gas channel. According to Michaelis-Menten kinetics [39], the specific activity of D_2/H^+ exchange (V) is:

$$V = \frac{[D_2]V_{\max}^{H_2O}}{[D_2] + K_M^{D_2}} \quad (2.2)$$

$K_M^{D_2}$ is the Michaelis constant for the substrate and is equal to:

$$K_M^{D_2} = \frac{k_{cat}^{H_2O} + k_{-1}^{D_2}}{k_{+1}^{D_2}} \quad (2.3)$$

and $V_{\max}^{H_2O}$ is the specific D_2/H^+ activity at infinite D_2 concentration, from which $k_{cat}^{H_2O}$ can be calculated by multiplying the former value by the Hase molecular weight.

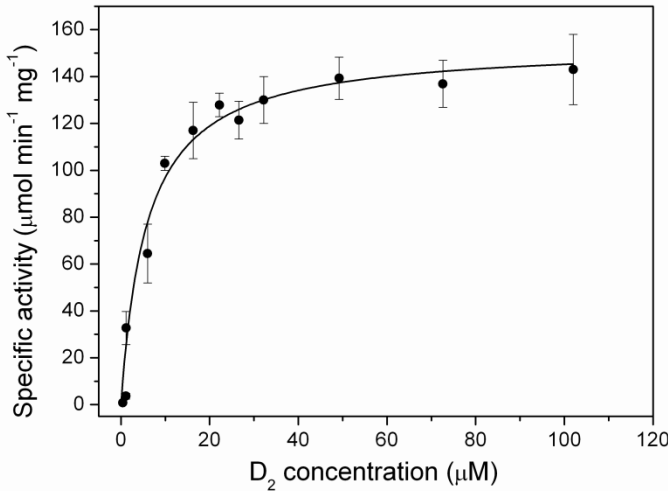


Figure 2.4 Dependence of the specific activity of HD + H₂ production of DvH NiFeSe Hase on the initial concentration of D₂. The solid line corresponds to the best fit to equation 2.2.

The dependence of the specific activity of HD + H₂ production of the NiFeSe Hase, which is equivalent to the enzymatic uptake of D₂, on the initial

concentration of D_2 is represented in Figure 2.4. By fitting the experimental data to equation (2) we determined the Michaelis constant for D_2 ($K_M^{D_2} = 6 \pm 1 \mu\text{M}$) and the maximal specific activity ($V_{\max}^{H_2O} = 154 \pm 7 \mu\text{mol min}^{-1} \text{mg}^{-1}$).

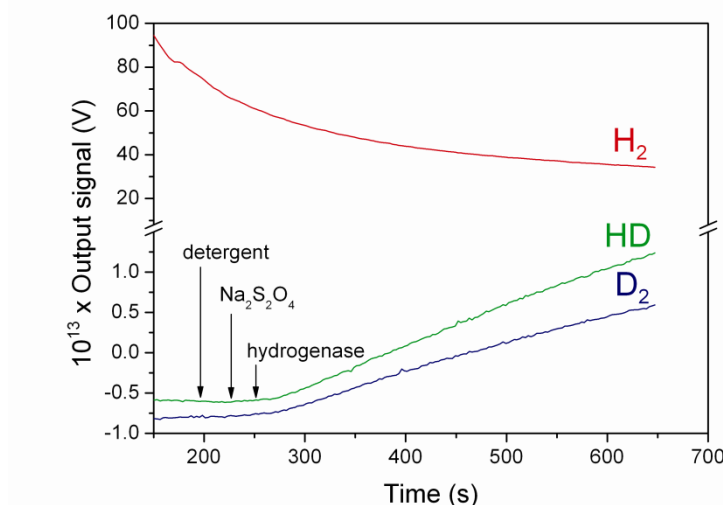


Figure 2.5 H_2/D^+ exchange activity of *DvH* NiFeSe Hase followed by mass spectrometry. Measurement done at 37°C in 20 mM phosphate buffer in D_2O , pD 7.0 and saturated with H_2 . The arrows indicate additions of 0.01% DDM, 0.1 mM sodium dithionite and 0.25 nM Hase.

The isotope exchange activity of the Hase was also measured in H_2/D_2O . Figure 2.5 shows that in the H_2/D^+ assay the rate of single exchange production is now higher than that of the double exchange production. On average and at pD 7.0, the initial rate of HD-production was 2 times faster than that of H_2 -production. It is interesting to compare the ratio of single exchange production to double exchange production in H_2O versus D_2O . Table 2.1 presents a comparison between the H_2/HD ratios (in H_2O) with the corresponding values for D_2/HD (in D_2O), for different Hases at the optimum pH value. It is

noteworthy that there is a small or no isotope effect at all for anaerobic NiFe Hases (such as those from *Thiocapsa roseopersicina* (*Tr*) and *Dg*, whereas it shows to be significant for the NiFeSe Hase. A similar effect was reported for the aerobic H₂-sensor protein of *Re*, which was attributed to a cage effect around the active site [40].

Table 2.1 Ratio of single exchange production to double exchange production of different Hases.

Hase	<i>DvH</i> NiFeSe ^a	<i>Dg</i> ^b	<i>Re</i> H ₂ -sensor ^c	<i>Tr</i> ^d	<i>R.</i> <i>capsulata</i> ^e	<i>P.</i> <i>denitrificans</i> ^e
HD/H ₂ (H ₂ O)	0.4	2.5	0	2.5	0.4	0.4
HD/D ₂ (D ₂ O)	2.0	5.0	0.8	2.5	1.4	0.6

^a Measured in this work.

^b De Lacey, A. L.; Fernandez, V. M. (unpublished results).

^c ref 40.

^d refs 41 and 42.

^e ref 29

^f ref 43.

The dependence of the H₂/D⁺ exchange activity of the NiFeSe Hase on the substrate concentration was also studied (Figure 2.6). By fitting the experimental data to Michaelis-Menten kinetics, as performed in the D₂/H⁺ assay, we determined the Michaelis constant for H₂ ($K_M^{H_2} = 34 \pm 3 \mu\text{M}$) and the maximal specific activity in D₂O ($V_{\text{max}}^{D_2O} = 142 \pm 7 \mu\text{mol min}^{-1} \text{mg}^{-1}$). Therefore, there is no significant isotope effect in the maximal specific exchange activity, but the K_M for H₂ is more than 5 times higher than for D₂. This means that there is a significant isotope effect in the affinity of this Hase for the substrate.

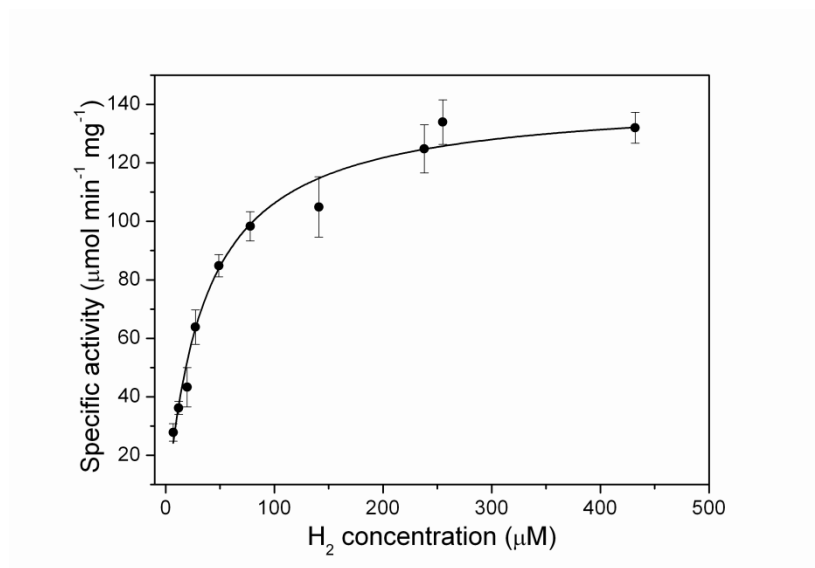


Figure 2.6 Dependence of the specific activity of HD + D₂ production of *DvH* NiFeSe Hase on the initial concentration of H₂. The solid line corresponds to the best fit to equation 2.2.

2.3.1 Molecular dynamics (MD) simulations

Previous MD studies concerning the diffusion of H₂ in the NiFeSe Hase from *Db* revealed the existence of an alternative pathway for the access of H₂ to the active site, which is absent in NiFe Hases [14]. The comparison of the crystal structures indicated the existence of the same cavity in the NiFeSe Hase of *DvH* [28]. We hypothesised that the existence of this cavity may explain the strong isotope effect detected experimentally. In order to get molecular insights into this phenomenon, we have performed molecular dynamics simulations of the diffusion of H₂ and D₂ from the outside to the inside of the enzyme.

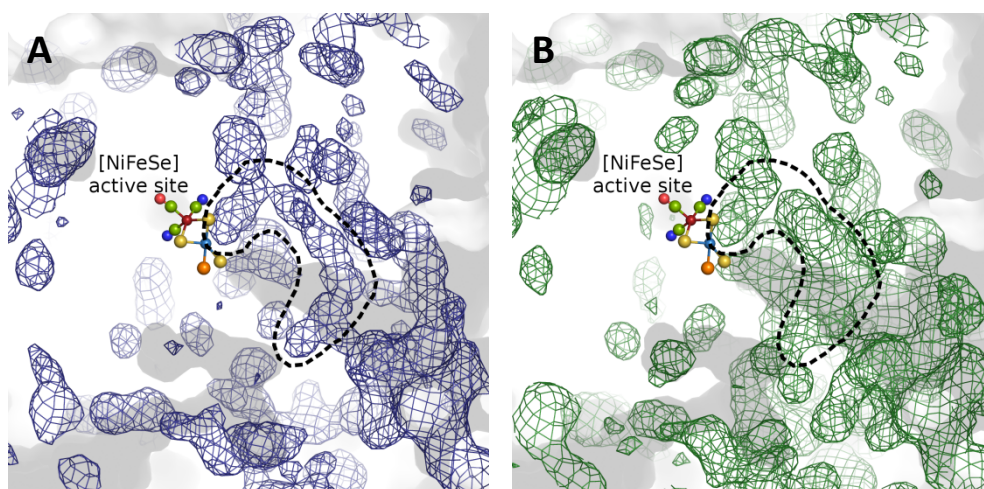


Figure 2.7 Probability density maps of H_2 (A) and D_2 (B) in the NiFeSe Hase from *DvH*. The probability density contour is depicted as a mesh. The crystallographic structure is represented as a surface. The metal centres are depicted as balls and sticks. The probability density maps are calculated using the positions of the H_2 and D_2 centre of mass in every 5 ps of the last 20 ns of our ten simulation replicates, which is the period when we considered that the amount of H_2/D_2 inside the protein is stable. The cavity near the active site is highlighted by a dashed line.

The results reveal that the diffusion of H_2 in the NiFeSe Hase from *DvH* is similar to the diffusion observed previously in *Db* [14]. H_2 uses the channels that are visible on the crystal structure to enter the protein, but the access to the active site is mainly done via the alternative cavity, as can be seen in the probability density maps depicted in Figure 2.7A. D_2 also uses the same cavity, but the probability of finding D_2 in the cavity is higher (Figure 2.7B), which is likely due to the fact that D_2 diffuses slower and stays longer in these cavities. The high accumulation of D_2 in comparison to H_2 is also visible in the radial distribution function, which has a large peak for D_2 at 8 Å (Figure 2.8).

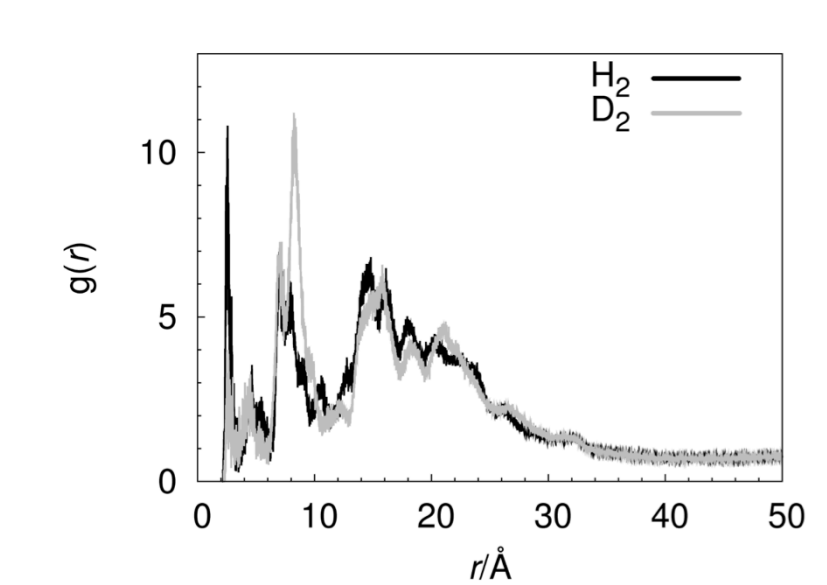


Figure 2.8 Radial distribution functions of H_2 and D_2 with respect to the Ni in the NiFeSe Hase from *DvH*. The density of H_2/D_2 was calculated as described in Figure 2.7.

2.4 DISCUSSION

Our results show that there is a strong dependence of the D_2/H^+ activity of the NiFeSe Hase on pH. The optimum of activity at pH 6 suggests that proton exchange at the active site is rate-limited by a protonable group of pK_a approximately 6, as this behaviour is expected for a catalytic mechanism that involves deprotonation and reprotonation of the dihydrogen complex intermediate [22]. The reported pH optimum for the NiFe Hase from *Dg* in the same assay was 7.8, whereas for another NiFeSe Hase (from *Db*) was 4.5 [44]. Therefore, it is tempting to explain the shift of the pH optima towards acidic values for NiFeSe Hase by the replacement of one the terminal cysteines of the active site by selenocysteine, as the latter amino acid has a lower pK_a , and it is

generally considered that this Ni ligand is involved in proton transfer during the catalytic cycle [6]. Nevertheless, it must be taken into account that several other basic sites of the enzyme may also be rate-limiting the catalytic process.

The main difference in the D_2/H^+ activity between NiFeSe and NiFe Hases is that the former produce the double exchange product (H_2) faster than the single exchange product (HD) from time zero, whereas the latter do the contrary. This catalytic property of NiFeSe Hases has been explained either by an effect of the Se atom coordinated to the active Ni [41, 43, 45, 46] or by a “cage effect” of the protein structure surrounding the active site [40, 46, 47]. In the first case it has been proposed that the lower electronegativity of Se compared to S increases the electron donation to the bimetallic active site, which is in agreement with the lower vibrational frequencies measured by FTIR for the active site’s CO ligand [48], favouring the isotope exchange of D^- after heterolytic cleavage of D_2 and before formation of HD at the active site [41, 43, 45], as shown in Figure 2.1 B (step a). Alternatively, it has been proposed that double exchange product formation involves successive reactions of the formed HD intermediate due to its accumulation near the active site, as shown in Figure 2.1B (step b). This effect would be caused by slow release of the intermediate to the solution due to a steric effect of the protein structure surrounding the active site, the named “cage effect” [40, 46, 47]. In this work we clearly show that the initial rates of D_2 formation are only faster than the HD formation at pH values around the pH optimum, whereas at the most lower and higher pH values the rate of HD formation is faster. If the double exchange formation would involve two proton transfer steps in the same catalytic cycle, higher H_2/HD ratio would be expected at the lower pH values because more protons are available. This is the case observed for D_2/H^+ exchange catalysed by Rh

complexes, for which a catalytic mechanism was proposed where two successive protonation steps take place in the same cycle at low pH [22]. Carriker *et al.* argued that if double exchange formation would require release of the HD intermediate from the catalyst followed by recoordination, then the double-exchange/single-exchange ratio would increase at the pH values where the catalyst turnover is higher [22]. This behaviour is exactly what we observe for DvH Hase in Figure 2.2. This difference in catalytic behaviours between the organometallic catalyst and the biocatalyst is in consonance with the bimetallic active site of the Hase being buried inside the protein structure [28].

The high isotope effect measured in the H^+/D^+ exchange activity also favours that there is a considerable “cage effect” in the catalytic mechanism of the NiFeSe Hase. The higher single-exchange/double-exchange ratio in the $\text{H}_2/\text{D}_2\text{O}$ system can be explained by more accumulation of D_2 product around the active site due to its slower release towards the solution, thus lowering the chances of the HD intermediate molecules entering into a second catalytic cycle. The opposite effect is expected in the $\text{D}_2/\text{H}_2\text{O}$ system, in which more HD would accumulate around the active site than the H_2 product, thus increasing the probability of successive exchange reactions of the HD intermediate. This hypothesis is in agreement with the higher rate of double exchange measured in the $\text{D}_2/\text{H}_2\text{O}$ system. The isotope effect changes considerably for different types of Hases, as shown in Table 2.1. There is an extreme effect for the H_2 -sensor from *Re* Hase, which was attributed also to a “cage effect” due to the presence of bulky amino acid residues surrounding the access of the H_2 channel to the active site [40]. Other Hases have small or no isotope effects at all. These results suggest that the protein structure surrounding the active site in each Hase modulates the rates of transport of dihydrogen molecules to/from the

active site. In fact, a recent computational study has compared H₂ transport within the NiFe Hase from *Dg* with the NiFeSe Hase from *Db* [14]. This work concluded that differences in some of the amino acid residues surrounding the active site cavity change the access routes of H₂ to the active site. Furthermore, the molecular dynamics simulations gave a higher accumulation of the substrate around the active site in the NiFeSe Hase [14]. In the present work the MD simulations of the diffusion of H₂ and D₂ in the *DvH* NiFeSe Hase showed that both the H₂ and D₂ molecules use an alternative channel to access the active site, similarly as found in the *Db* enzyme for H₂ [14]. The MD simulations revealed higher accumulation of D₂ in comparison with H₂ in the referred channel of the *DvH* Hase. This difference is totally related with the different masses of the two species, since it was the only parameter changed. Despite not having simulated HD, it is reasonable to assume that it would have behaviour between these two extreme situations, accumulating more than H₂ but less than D₂. Therefore, the MD simulations are in agreement with the experimental H/D exchange activity data.

It is quite possible that the quite different single/double exchange ratios measured for different Hases (Table 2.1) are related to their structural differences in the connection of the active site cavity with the hydrophobic channels through which substrates and/or products are transported. It is expected that the larger the gas cavity next to the active site is, the more product molecules will accumulate next to the active site [14], and the “cage effect” in the catalytic activity will be larger.

The strong isotope effect in the K_M for the substrate is also in agreement with the “cage effect” on the Hase activity. The results of the MD simulations also support a higher affinity of D₂ to the protein when compared with H₂, given

that the higher accumulation of D₂ near the active site can be interpreted as a higher affinity and, consequently, a lower K_M . According to equation (3), the K_M depends on the k_{cat} and on the dissociation constant of the substrate (k_{-1}/k_{+1}). As there is no significant isotope effect in the V_{max} values measured, the large increase of the measured K_M for H₂ is due to an increase of its dissociation constant from the active site compared to D₂. Therefore, this result is in agreement with a higher rate of release of H₂ from the active site to the solution than for D₂ ($k_{-1}^{\text{H}_2} > k_{-1}^{\text{D}_2}$). It has been reported that the measured $K_M^{\text{H}_2}$ of a NiFe Hase is very sensitive to mutations of amino acid residues lining the H₂-transport channel, increasing up to two orders of magnitude when the putative H₂ channel was made narrower by introducing more bulky amino acids [38, 49]. Thus, the high isotope effect in the K_M for the substrate we have measured in this work can also be correlated with the structural differences of the H₂-transport channels to/from the active site in NiFeSe Hases [14].

In summary, the results of this work indicate that in the catalytic mechanism of Hases the double exchange of protons requires the unbinding of the HD intermediate from the bi-metallic active site followed by its re-coordination before it is released towards the solution, and not the exchange of two protons in the same catalytic cycle as some inorganic complexes do. Moreover, both experimental and computational results are in agreement with a “cage effect” of the protein structure surrounding the active site, modulating its catalytic function. These conclusions have implications in the design of functional biomimetic catalysts, showing that the presence of a supramolecular structure surrounding the bimetallic centre is important for modulating the heterolytic oxidation/production of H₂.

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Chapter 2

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CHAPTER 3

Redox State-Dependent Changes in the Crystal Structure of NiFeSe Hydrogenase from *Desulfovibrio vulgaris* Hildenborough

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ABSTRACT

Hases are enzymes that can potentially be used in bioelectrical devices or for biological hydrogen production, the most studied of which are the NiFe type. Most NiFe Hases are inactivated by oxygen and the few known O₂-tolerant enzymes are hydrogen-uptake enzymes, unsuitable for hydrogen production, due to strong product inhibition. In contrast, the NiFeSe Hases, where a selenocysteine is bound to the nickel, are very attractive alternatives because of their high hydrogen production activity and fast reactivation after O₂ exposure. Here we report five high-resolution crystallographic 3D structures of the soluble form of the NiFeSe Hase from *DvH* in three different redox states (oxidized as-isolated, H₂-reduced and air re-oxidized), which revealed the structural changes that take place at the active site during enzyme reduction and re-oxidation. The results provide new insights into the pathways of O₂ inactivation in NiFe, and in particular NiFeSe, Hases. In addition, they suggest that different enzymes may display different oxidized states upon exposure to O₂, which are probably determined by the protein structure.

3.1. INTRODUCTION

At a critical stage of the fossil fuel crisis and global warming, hydrogen is drawing increased attention as an energy carrier with great potential to become an environment-friendly fuel in the future. However, H_2 production still relies on the thermocatalytic transformation of fossil fuels, because the apparently simple reaction of producing H_2 from H^+ and e^- is in fact very difficult to achieve. Hases are the natural enzymes for the production and oxidation of H_2 in ambient conditions that are far more efficient than Pt-based catalysts, and biological H_2 production offers a promise for H_2 generation using clean technologies and renewable sources [1-3].

Hases are classified according to the metal content of their active site: di-iron (FeFe), nickel-iron (NiFe), and iron-sulphur cluster free (Fe) Hases [4, 5]. Among these, the NiFe Hases are the most common and most studied enzymes. Based on a phylogenetic and functional analysis, the NiFe proteins can be divided into four physiological groups: periplasmic H_2 -uptake Hases, cytoplasmic H_2 -uptake Hases, cytoplasmic Hases with additional subunits (bidirectional Hases) and energy-converting membrane-bound Hases [4]. The minimum biological unit of NiFe Hases is composed of two subunits: the small electron transferring subunit contains three covalently bound iron–sulphur clusters, and the large subunit harbours the bimetallic active site. In the catalytic centre, the Ni and Fe atoms are bridged by two cysteines and two other cysteines coordinate the Ni atom.

A major difficulty in the biotechnological application of Hases derives from the fact that O_2 can react with the catalytic centre, inactivating the enzymes [6]. Whereas the FeFe Hases are irreversibly damaged by O_2 , in the

NiFe enzymes the inactivation is reversible. In H₂-oxidizing aerobic bacteria such as *Ralstonia eutropha* (*Re*), more O₂ tolerant enzymes are present [2, 7-9]. However, these enzymes are designed for H₂ oxidation and exhibit very low H₂ production activities, as well as strong product inhibition by H₂ [10]. The membrane-bound NiFe uptake Hases from *E. coli* and *A. aeolicus* (*Aa*) also display a high catalytic activity for H₂ oxidation in air [11, 12]

The NiFe Hases display several redox states. The inactive oxidized states are usually a mixture of Ni-A (unready) and Ni-B (ready) states, both of which are paramagnetic [13]. In the Ni-B state, the enzyme is quickly reactivated upon reduction, whereas the activation of an enzyme in the Ni-A state requires a much longer time period. The crystallographic structures of oxygen-sensitive NiFe Hases showed that in the oxidized state an oxygen species is present, bridging the NiFe site (reviewed in [14]). It is currently believed that this ligand is a hydroxide (OH⁻) in the Ni-B state [15], whereas the structure of the Ni-A state is still a matter of debate, as a hydroperoxy (OOH⁻) ligand was reported for the NiFe enzymes from *Desulfovibrio* spp. (reviewed in [16]), but in Av Hase a structure of the Ni-A state revealed the presence of only a mono-oxide bridging ligand [17].

Extensive research has been carried out on increasing the O₂-tolerance of NiFe Hases. Studies on O₂-sensitive Hases have focused mainly on restricting O₂ access to the active site by narrowing the hydrophobic gas channels leading to it (see e.g., [12, 15, 16]), whereas studies on O₂-tolerant Hases have aimed at elucidating the structural basis of O₂ tolerance (see e.g., [12, 18-22]). The crystal structures of three O₂-tolerant NiFe Hases have been recently reported [19, 22, 23], and the proximal iron-sulphur cluster was identified as the structural element responsible for O₂-tolerance. This cluster is coordinated by six cysteine

residues instead of the usual four in O₂-sensitive Hases, and one of the sulphur iron ligands is replaced by a cysteine sulphur atom. This unprecedented [4Fe-3S] cluster is proposed to be capable of undergoing two redox transitions (instead of only one in standard [4Fe-4S] clusters), and thus prevent the enzyme from reaching the Ni-A state upon exposure to O₂ [20].

The NiFeSe Hases are a sub-group of the NiFe enzymes where a selenocysteine (Sec) residue replaces a cysteine as a terminal Ni ligand (reviewed in [24]). The NiFeSe Hases show some unique properties including a higher H₂-production than H₂-oxidation activity [24-27]. Their oxidized states do not display the characteristic Ni-A or Ni-B inactive states after aerobic isolation, typical of O₂-sensitive NiFe Hases, and they become active immediately upon reduction. Although it was shown that the NiFeSe enzymes from *Db* and *DvH* react with oxygen to produce an inactive state, both enzymes display a very fast reactivation requiring a low redox potential [26, 28], suggesting different oxidized species from those formed in standard NiFe Hases. In addition, the *Db*, NiFeSe Hase exhibits less inhibition by H₂ than standard NiFe Hases, and it can sustain H₂ production in the presence of low O₂ levels [26]. Overall, these properties make NiFeSe Hases very interesting targets for H₂ production. This was recently demonstrated in a system using the *Db* enzyme for photocatalytic H₂ production under non-strict anaerobic conditions [29]. In another application, we observed good electrocatalytic currents for H₂ production from the *DvH* enzyme bound to a gold electrode [30]. In contrast to a wealth of studies on the O₂ inactivation of NiFe Hases [13, 31-33] very few studies have addressed the same topic in NiFeSe Hases. However, this is an important matter for future biotechnological applications of these enzymes, since they share some of the attractive properties of the O₂-tolerant enzymes (absence of Ni-A

species and fast reactivation) and consequently appear to have some level of protection to O₂ exposure [34], while exhibiting much higher H₂ production activities.

The NiFeSe Hase from *DvH* is an example of an enzyme with a high H₂ production activity [25]. *DvH* is an anaerobic organism containing several different Hases of the FeFe and NiFe types, and it can use H₂ as an energy source, or grow fermentatively with H₂ production. The NiFeSe Hase is the major Hase expressed in *DvH* when Se is available [35], reflecting its more efficient properties. This enzyme contains a lipidic group bound to the N-terminus of the large subunit that attaches it to the cellular membrane [36]. A soluble form without the lipidic group was also purified, which displayed lower activity [35]. Recently, we determined the crystal structure of the as-isolated oxidized soluble form of the NiFeSe Hase [37]. This was the first structure of an oxidized NiFe Hase that does not contain an oxide bridging ligand at the active site, confirming it does not form the Ni-A or Ni-B inactive states typical of oxygen-sensitive NiFe Hases. An exogenous sulphur atom is present instead, bound to Ni and Se, and causing a side-chain conformation change in the Sec that effectively blocks O₂ access to Ni. However, evidence for O₂ damage to the enzyme was revealed by the fact that the terminal Cys ligand of the Ni was found oxidized to a sulphinate state and the proximal [4Fe-4S] cluster was partially oxidized to [4Fe-3S-3O].

In this work we present five high-resolution crystallographic 3D structures of the soluble form of the *DvH* NiFeSe Hase in several redox states (oxidized as-isolated, H₂-reduced and air re-oxidized), revealing the structural changes that take place at the active site during enzyme reduction and re-oxidation. Our results demonstrate the involvement of the proximal [4Fe-4S]

cluster in enzyme inactivation after O₂ attack, and the much higher resolution of the oxidized state form obtained in the present work allowed the unambiguous assignment of the structure and stereochemistry of the modified cluster as a [4Fe-4S-3O] centre. This oxidative modification is reversed upon reduction, whereas the terminal cysteine linked to the Ni atom is irreversibly oxidized to sulphinate by O₂ attack during the purification and/or crystallization stages of the enzyme. These structural modifications differ from the oxidized states of the NiFe Hases described to date, bring new insights into this NiFe Hase superfamily, and are fundamental to understand their properties and for the correct design of devices using these enzymes for H₂ production, particularly in the case of the NiFeSe enzymes.

3.2. MATERIALS AND METHODS

3.3.1 Protein production, purification and enzymatic activity assay

The soluble form of *DvH* NiFeSe Hase, lacking the first 11 residues of the large subunit and the lipidic group, was isolated and purified as previously described by [38]. H₂ production activity was determined by gas chromatography at pH 7.6, as described in reference [25], with dithionite as electron donor and in the presence of methyl viologen. The soluble form of *DvH* NiFeSe Hase was diluted 500x in 20 mM Tris buffer pH 7.6 and incubated at 37°C for 45 minutes.

To circumvent the low and non-reproducible yield of soluble *DvH* NiFeSe Hase using the protocol previously described in reference [38], this protein was also produced by incubation of the membrane form with a lipase from *Rhizopus*

niveus (Sigma). The assay consisted in mixing 50 μ M of the membrane form of DvH NiFeSe Hase with 5 μ g of lipase, in a 20 mM Tris-HCl pH=8.0 buffer, at 37°C for 12h. After that time, a yield of almost 99% soluble NiFeSe Hase was obtained. The sample obtained was checked for Hase activity and was further purified on a Pharmacia Resource Q column, equilibrated with 20 mM Tris-HCl pH=7.6 buffer, applying a stepwise gradient of 1M NaCl. The eluted fraction containing pure soluble DvH NiFeSe Hase was washed and concentrated to 11 mg/mL in 20 mM Tris-HCl pH 7.6.

3.3.2 Crystallization and X-ray diffraction data collection

Crystals of the native soluble form of DvH NiFeSe Hase were obtained aerobically at room temperature, as previously described [38]. Crystals of the as-isolated, oxidized form were cryoprotected using a solution containing 20% glycerol, 20.5% PEG 1500 (w/v) and 0.1 mM Tris-HCl pH 7.6, flash-cooled in N₂(l) and transferred to a storage dewar. Reduced crystals were prepared from crystals of the oxidized form. The crystals were first transferred to a new drop containing 7 μ l of the cryoprotecting solution, to which were added 1 μ l of 10 mM methyl-viologen and 50 mM Tris-HCl pH 7.6 buffer. Then, the drop was kept in an anaerobic environment, 15 % H₂ and 85% N₂, until the solution and the crystals became completely blue, an indication of their reduced state. The drop containing the reduced crystals was then transferred to an anaerobic glove box, and some of the reduced crystals were harvested, flash-cooled in N₂(l) and stored. The drop with the remaining crystals was subsequently removed from the anaerobic environment, and equilibrated. The re-oxidized crystals were obtained by air oxidation of reduced crystals for 24h or 48h at

20°C, which were then harvested, flash-cooled in N₂(l) and stored. All solutions used in this procedure were degassed with nitrogen prior to their use, and stored under anaerobic conditions. The crystals of oxidized, reduced and re-oxidized *DvH* NiFeSe Hase were transferred to a dry shipper and sent to the PXIII beamline at the Swiss Light Source (SLS) of the Paul Scherrer Institute in Villigen, Switzerland, for data collection. Diffraction data sets were collected from one oxidized (Ox1), one reduced (Red1) and one re-oxidized for 24h (ReOx24) single crystals of *DvH* NiFeSe Hase. The diffraction pattern of the crystal of re-oxidized for 48h was multiple and no useful data could be collected.

Crystals of soluble *DvH* NiFeSe Hase prepared from the membrane form by incubation with a commercial lipase were obtained using the sitting-drop vapour diffusion method. 1 µL of a reservoir solution containing 16% PEG 8000 (w/v) and 0.05 M KH₂PO₄ pH 4.1 was mixed with an equal volume of a solution composed of 11 mg/mL protein in 20 mM Tris–HCl buffer pH 7.6, and equilibrated against a 500 µL reservoir. Crystals appeared within 5 days. One crystal of the oxidized form was cryoprotected using a solution containing 20% glycerol, 16% PEG 8000 (w/v) and 0.05 M KH₂PO₄ pH 4.1, flash-cooled in N₂(l) and transferred to a storage dewar. Reduced crystals were obtained from crystals of the oxidized form by incubation in a drop containing 5 µL 16% PEG 8000, 0.05 M KH₂PO₄ pH 4.1 and 20% glycerol, to which were added 0.4 µL of 10 mM methyl-viologen and 0.05 M KH₂PO₄ pH 4.1 buffer, previously flushed with N₂. Subsequently, the drop was kept for 30 min. under an anaerobic environment (15% H₂ and 85% N₂) and sodium dithionite was used as reducing agent. Small amounts were added until the solution and the crystals turned blue, taken as an indication of their reduced state. The reduced crystals were

quickly harvested, flash-cooled in $N_2(l)$ and stored. These crystals of oxidized and reduced *DvH* NiFeSe Hase were transferred to a dry shipper and sent to the ID29 beamline at the European Synchrotron Radiation Facility (ESRF) for data collection. Diffraction data sets were collected from one oxidized (Ox2) and one reduced (Red2) single crystals of *DvH* NiFeSe Hase. No crystals of re-oxidized protein could be obtained under these conditions, as they re-dissolved a few hours after reduction and removal from the anaerobic environment.

For all datasets, the wavelength was chosen at (SLS) or near (ESRF) the absorption edge K of the selenium atoms. The diffraction images were processed with XDS [39] and conversion of intensities to structure factor amplitudes was carried out with CTRUNCATE in the CCP4 program suite [40]. The data collection and processing statistics are listed in Table 3.1.

Table 3.1 - Data collection and processing statistics

	Oxidized 1 (Ox1)	Oxidized 2 (Ox2)	Reduced (Red1)	Reduced (Red2)	Re-oxidized (ReOx24)
Beamline	SLS PXIII	ESRF ID29	SLS PXIII	ESRF ID29	SLS PXIII
Detector	MAR 225 CCD	PILATUS 6M	MAR 225 CCD	PILATUS 6M	MAR 225 CCD
Wavelength (Å)	0.9796	0.9762	0.9796	0.9762	0.9796
Data Processing	XDS/CCP4	XDS/CCP4	XDS/CCP4	XDS/CCP4	XDS/CCP4
Space Group	$P2_12_12_1$	$C2$	$P2_12_12_1$	$P3_121$	$P2_12_12_1$
Unit cell parameters (Å,°)					
a	72.17	106.69	72.30	61.80	72.27
b	97.35	63.35	96.99	61.80	97.63
c	102.97	109.74	103.86	339.30	103.35
α	90	90	90	90	90
β	90	105.42	90	90	90
γ	90	90	90	120	90
Resolution (Å) ^a	45.5-1.50 (1.54-1.50)	53.9-1.33 (1.41-1.33)	45.8-1.95 (2.07-1.95)	56.6-1.82 (1.93-1.82) ^b	44.1-1.80 (1.91-1.80)
Nr. observations	830460 (41625)	487017 (77560)	194894 (29857)	275858 (33355)	250342 (39571)
Unique reflections	114933 (7556)	159127 (25456)	53472 (8247)	67904 (9884)	64222 (10480)
Multiplicity	7.2 (5.5)	3.1 (3.0)	3.6 (3.6)	4.1 (3.4)	3.9 (3.8)
Completeness (%)	98.6 (88.5)	98.3 (97.5)	99.0 (95.8)	97.7 (89.9)	94.0 (96.3)
R-merge (%) ^c	9.0 (58.9)	3.5 (50.5)	12.8 (49.2)	3.7 (7.9)	6.6 (34.0)
$\langle I/\sigma(I) \rangle$	17.5 (3.0)	14.2 (1.7)	11.1 (3.3)	23.3 (9.3)	16.2 (4.1)
R-meas (%) ^d	9.8 (65.1)	4.2 (61.3)	15.0 (57.7)	4.2 (9.2)	7.6 (39.2)
Wilson B (Å ²)	9.9	15.3	13.9	15.6	14.1
Z^e	1	1	1	1	1
Estimated V_m^f	2.18	2.12	2.16	2.22	2.20
Estimated SolvCont (%) ^f	43.6	42.0	43.0	44.5	44.0
PDB accession code	3ze6	3ze9	3z37	3zea	3ze8

^a Values in parentheses refer to the highest resolution shell. ^b The high resolution limit was restricted due to spatial overlaps caused by the long cell axis in an unfavorable orientation. ^c R-merge = merging R-factor, $(\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|) / (\sum_{hkl} \sum_i I_i(hkl)) \times 100$ % ^d R-meas = redundancy independent R-factor, $\sum_{hkl} [N/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / (\sum_{hkl} \sum_i I_i(hkl)) \times 100$ % [70]. For each unique Bragg reflection with indices (hkl), I_i is the i-th observation of its intensity and N its multiplicity. ^e Nr. molecules in the asymmetric unit. ^f According to Matthews coefficient [71]

3.3.3 Structure determination and refinement

The five crystal structures of *DvH* NiFeSe Hase in each of the three redox forms were determined by the molecular replacement method with PHASER [41] via the CCP4 Graphics User Interface [42]. The coordinates of the protein chains of the large and small subunits of the previously published crystal structure of *DvH* NiFeSe Hase (PDB 2wpm) [38] were used as search models, after removal of all non-protein atoms. Also, the side-chain of the Sec residue was truncated after its C^β atom prior to the calculations.

The structures were refined with PHENIX [43]. Each PHENIX refinement stage consisted of three cycles with refinement of positional coordinates, individual isotropic atomic displacement parameters for all non-hydrogen atoms and also refinement of the anomalous dispersion parameters for the Se atom, followed by model inspection and editing with Coot [44] against sigmaA-weighted $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ electron density maps. Water molecules were added with PHENIX and checked with Coot. In the Ox1, Ox2 and Red2 structures, hydrogen atoms in calculated positions were added to the structural model and included in the refinement. In the Ox2, Red1, Red2 and ReOx24 structures, TLS (translation-libration-screw) rigid body refinement of atomic displacement parameters was carried out, followed by refinement of individual isotropic B-factors. For each crystal structure, the TLS groups were parameterized using the TLS Motion Determination (TLSMD) server [45]. In the Ox1 and ReOx24 structures, 5 incomplete chains of 3-[dodecyl(dimethyl)ammonio]propane-1-sulfonate detergent (SBC), used in the protein purification, were identified in the electron density maps and included in the refinement. The quality of the five refined crystal structures was assessed

with MolProbity [46] as included in PHENIX, and the corresponding refinement and validation statistics are presented in Table 3.2. Occupation factors were refined for three Sec489 conformers in the Ox1 and Ox2 structures, two Sec489 conformers in the Red2 and ReOx24 structures, and two forms of the proximal [Fe-S] cluster in the Ox1 structure. The final occupation factors are listed in Table 3.3. Figures 3.2 through 3.9 were generated using PyMOL Molecular Graphics System, Version 1.4.1 (Schrödinger, LLC).

Chapter 3

Table 3.2 Refinement statistics

Dataset	Oxidized 1, Ox1	Oxidized 2, Ox2	Reduced, Red1	Reduced, Red2	Reoxidized, ReOx24
Resolution limits (Å) ^a	45.5-1.50 (1.52-1.50)	52.9-1.33 (1.35-1.33)	45.8-1.95 (1.97-1.95)	56.6-1.82 (1.84-1.82)	44.1-1.80 (1.82-1.80)
R_{work} ^b	0.135 (0.240)	0.131 (0.302)	0.153 (0.236)	0.124 (0.146)	0.135 (0.200)
No. reflections in working set ^c	210208 (5740)	286382 (8780)	96625 (2649)	106181 (2544)	111795 (3668)
R_{free} ^d	0.154 (0.231)	0.148 (0.307)	0.190 (0.247)	0.147 (0.166)	0.166 (0.244)
Nr. reflections in test set ^c	11120 (300)	15046 (451)	5092 (143)	5689 (131)	5953 (182)
Cruickshank DPI (Å) ^e	0.04	0.03	0.10	0.07	0.08
ML coordinate error estimate ^f	0.13	0.11	0.19	0.16	0.14
<i>Model composition and completeness</i>					
Regions omitted	1A-6A, 1B-14B	1A-4A, 1B-14B	1A-6A, 1B-14B	1A-4A, 1B-14B	1A-6A, 1B-14B
Disordered residues	35	34	7	15	16
Non-hydrogen protein atoms ^g	5993	6024	5857	5902	5901
Ligand/ion	40	38	34	35	35
Solvent molecules	700	741	734	637	639
Glycerol	12	-	-	-	-
Detergent (partial chains)	53	-	-	-	49
<i>Mean B values (Å²) ^h</i>					
Protein main-chain	10.9	19.2	12.5	16.3	13.6
Protein side-chain	14.1	22.3	15.2	20.1	16.7
Ligand/ion	9.2	15.3	11.9	14.3	14.6
Solvent	23.4	33.8	25.7	30.0	26.4
Glycerol	22.3	-	-	-	-
Detergent	31.0	-	-	-	33.3
<i>Model r.m.s. deviations from ideality</i>					
Bond lengths (Å)	0.012	0.012	0.009	0.010	0.009
Bond angles (°)	1.476	1.303	1.148	1.251	1.126
Chiral centres (Å ³)	0.091	0.085	0.075	0.075	0.076
Planar groups (Å)	0.008	0.008	0.006	0.007	0.005

Table 3.2 Refinement statistics (cont.)

<i>Model validation</i> ⁱ					
% Ramachandran outliers	0.4	0.4	0.1	0.4	0.1
% Ramachandran favoured	97.3	98.0	97.3	97.8	97.3
% Rotamer outliers	1.1	1.2	1.3	1.1	0.8
C ^β outliers	3	0	0	0	0
Clash score	6.4	4.9	5.3	4.4	5.4
PDB accession ID	3ze6	3ze9	3ze7	3zea	3ze8

^a Values in parentheses refer to the highest resolution shell; ^b $R_{\text{work}} = (\sum_{\text{hkl}} ||F_{\text{obs}}(\text{hkl})| - |F_{\text{calc}}(\text{hkl})||) / (\sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl})|) \times 100\%$; ^c The Bijvoet pairs were treated as separate reflections due to the refinement of the anomalous dispersion coefficients for the Se atom; ^d R_{free} is calculated as above from a random sample containing 5% of the total number of independent reflections measured. ^e DPI is the diffraction-component precision index of the refined atomic coordinates and is estimated from R_{free} by the formula $\text{DPI} = (N_i/n_{\text{obs}})^{1/2} \times C^{-1/3} \times R_{\text{free}} \times d_{\text{min}}$ [72], where N_i is the number of non-hydrogen atoms, n_{obs} is the number of reflections included in the refinement, C is the fraction completeness of those reflections and d_{min} the high-resolution limit of the diffraction data; ^f Maximum-likelihood estimate by PHENIX; ^g Including atoms in the alternate conformations of disordered groups of residues; ^h Calculated from isotropic or equivalent isotropic B-values; ⁱ Calculated with MolProbity [46]

3.3.4 Sequence analysis tools

Sequence data were recovered from the U.S. Department of Energy Joint Genome Institute website (<http://www.jgi.doe.gov>) and Integrated Microbial Genomes website (<http://img.jgi.doe.gov>). Multiple alignments were performed using ClustalX [47] and edited with GeneDoc (<http://www.psc.edu/biomed/genedoc>).

3.3. RESULTS AND DISCUSSION

3.3.1 Purification and enzymatic activity

We have never been able to obtain crystals from the native, membrane form of the *DvH* NiFeSe Hase, which contains a lipidic group at the N-terminus of the large subunit [36]. For this reason we used the soluble form for crystallographic studies, which does not have the lipidic group or the first 11 residues of the catalytic subunit. The soluble form exhibits a lower activity than the membrane form, supporting the notion that the lipidic group and/or the first 11 N-terminal residues are somehow important for retaining a high catalytic activity. The native soluble *DvH* NiFeSe Hase used for crystallization displayed a specific activity of 860 U mg⁻¹ for H₂ production, quantified by gas chromatography, whereas the H₂ production activity of the membrane form has been reported to be 6910 U mg⁻¹, under the best conditions [25]. To obtain more of the soluble protein we developed an *in vitro* enzymatic assay to promote cleavage of the N-terminal peptide, in which the membrane form was digested with a commercial lipase from *Rhizopus niveus* (Sigma-Aldrich). Complete conversion of the membrane NiFeSe Hase to active, soluble NiFeSe

Hase form (Figure 3.1) was obtained after a 12h incubation at 37°C. This procedure allowed the *in vitro* production of higher quantities of active soluble *DvH* NiFeSe Hase from its membrane form, which, after chromatographic purification, was also used for crystallization. The use of a lipase in an enzymatic assay such as described here has great potential application for the conversion of other lipoproteins into their respective soluble forms.

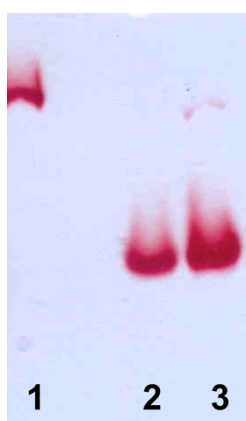


Figure 3.1. Activity-stained native PAGE. **1.** *DvH* membrane NiFeSe Hase; **2.** *DvH* soluble NiFeSe Hase; **3.** 50 μM of membrane *DvH* NiFeSe Hase incubated with 5 μg of lipase from *Rhizopus niveus* (10 mg.mL^{-1}), at 37°C, during 12h in 20 mM Tris-HCl pH 7.6 buffer.

3.3.2. Structure of *DvH* NiFeSe Hase

As previously reported [37], the overall 3D structure of the *DvH* NiFeSe Hase (Figure 3.2) is identical to that of a typical NiFe Hase: it is formed by two subunits, of which the larger contains the binuclear NiFe catalytic site, and the smaller contains three [Fe-S] clusters. Five crystal structures of the soluble *DvH* NiFeSe Hase in three redox forms were obtained. Three of these resulted from the crystallization of soluble enzyme isolated directly from cell extracts: the as-

isolated oxidized (Ox1), reduced (Red1) and re-oxidized (ReOx24) forms. Additionally, two more data sets were collected from crystals of soluble *DvH* NiFeSe Hase prepared from the membrane form by incubation with lipase: one oxidized (Ox2) and one reduced (Red2). All five crystal structures contain a single *DvH* NiFeSe Hase heterodimer in their asymmetric unit. The small and large subunits were designated chains A and B, respectively, and in the remainder of this text these chain identifiers are appended to the residue numbers to indicate which subunit they belong to.

All crystal structures reported herein were compared by superposition using the secondary structure matching algorithm [48] as implemented in the CCP4 suite. The as-isolated oxidized Ox1 structure was used as reference and each chain (A or B) was individually superposed. The results (Table 3.1) show that the largest differences occur between the small chains of the as-isolated oxidized Ox2, reduced Red1 and Red2 crystal structures and the corresponding chains in the as-isolated oxidized Ox1 and re-oxidized ReOx24 crystal structures. The differences are most pronounced in the N-terminal region of the small subunit that is visible in the electron-density maps (residues 7A-9A in Ox1, Red1 and ReOx24, 5A-9A in Ox2 and Red2) and in residues 29A-42A, which form a α -helix near the N-terminus. These differences are caused by the presence in the Ox1 and ReOx24 structures of four Sulfobetaine 3-12 detergent molecules, in two hydrophobic pockets. This detergent, also known as Zwittergent 3-12, was used in the purification of the *DvH* membranes, from which the soluble form (leading to crystals of Ox1, Red1 and ReOx24) was isolated [25, 38]. These chains are not present in the Ox2, Red1 and Red2 structures. The lipase digestion of the membrane protein and subsequent purification (leading to crystals that gave the Ox2 and Red2 structures) removed all traces of detergent

from the sample, as expected. However, since the crystals leading to the Ox1, Red1 and ReOx24 structures were obtained from the same protein batch of protein purified from cell membranes, it is not clear why there are no detergent molecules in the Red1 crystal structure. The larger hydrophobic pocket in the Ox1 and ReOx24 structures contains three Sulfobetaine 3-12 molecules and is located at the interface between the two subunits, but only residues 29A-42A were affected. The smaller pocket contains only one sulfobetaine 3-12 molecule, which influenced the conformation of the N-terminus of the small subunit. A fifth sulfobetaine 3-12 molecule is present in a third pocket located in the large subunit of Ox1 and ReOx24, but it does not introduce significant structural differences between the crystal structures.

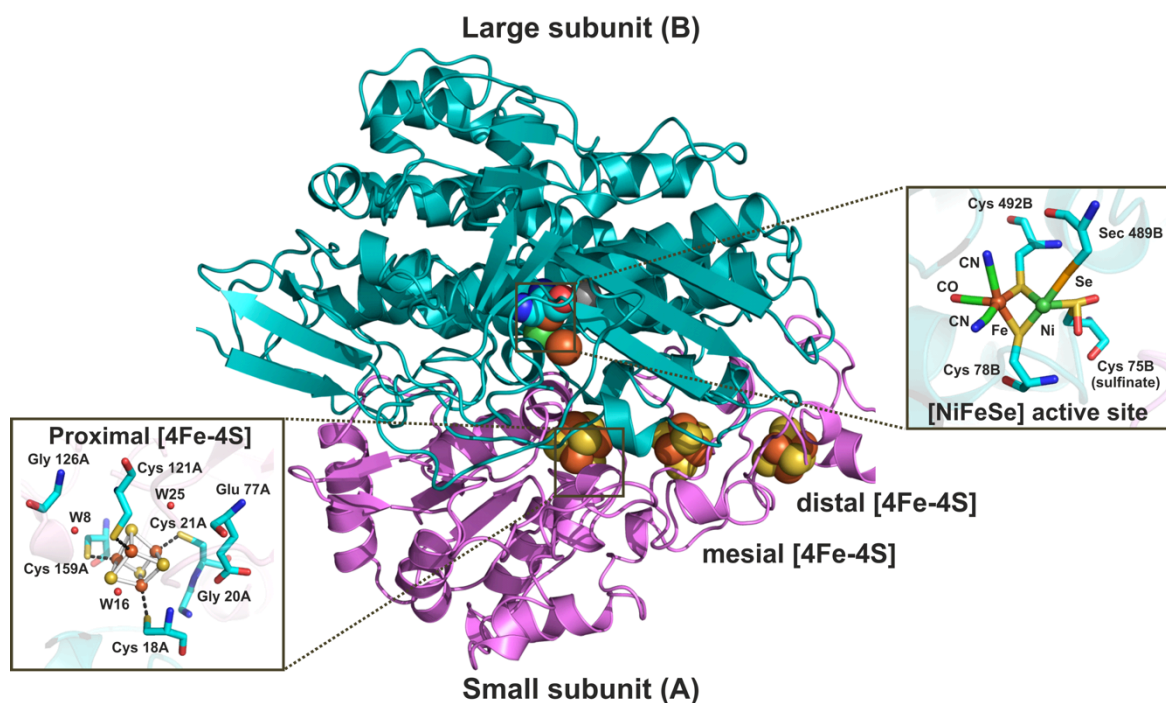


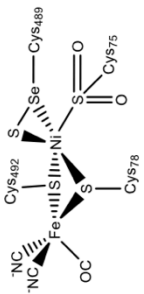
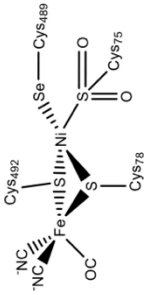
Figure 3.2 The overall structure of the NiFeSe Hase from *DvH* in the reduced state. The boxes show close-up views of the NiFeSe active site and the proximal [4Fe-4S] cluster, where the protein residues are drawn in stick representation, and the nickel, iron atoms and conserved water molecules are drawn as spheres. The proximal [4Fe-4S] cluster is drawn in ball-and-stick representation. Atom colours are cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel and gold for selenium. The additional H_2S molecule is represented in grey.

3.3.3. The structure of the active site in the different redox forms

The NiFe active site is anchored to the protein chain of the large subunit by two cysteine residues, 78B and 492B, which form thiolate bridges between the two metals, whereas the Ni atom is further ligated by Cys75B and Sec489B, and the Fe atom has the characteristic one CO and two CN⁻ diatomic ligands (Figure 3.2).

The Selenocysteine ligand to the Ni, Sec489B, is found to be present in three distinct conformers in the oxidized state, as previously reported [37], where in two of them the Se atom is bound to an exogenous sulphur atom (Table 3.3 and Figure 3.3). In both Sec489B conformers I and II the position of the S atoms is nearly the same, and the Se atom effectively shields the Ni atom in the active site from access by small diatomic molecules such as O₂ [37]. In conformer I both Se and S atoms coordinate Ni, while in conformer II only the S atom is bonded to Ni, and in conformer III no S atom is present and only Se binds Ni. Conformer III of Sec489 is the same as found for the equivalent Cys residue in the oxidized and reduced forms of NiFe Hases.

Table 3.3 - The active site and the proximal [Fe-S] cluster in the different redox states of the of *D. vulgaris* [NiFeSe] Hase

NiFeSe active site	Oxidized state		Reduced state		Reoxidized state	
						
Proximal [4Fe-4S]	Conformer I		Conformer III		Conformer II	
	Structure	Ox1	Ox2	Red1	Red2	ReOx24
	% Conformer I	74	73	-	-	-
	% Conformer II	16	13	-	12	38
Proximal [4Fe-4S]	Conformer I		Conformer III		Conformer II	
	Structure	Ox1	Ox2	Red1	Red2	ReOx24
	% [4Fe-4S-3O]	80	100	-	-	-
	% [4Fe-4S]	20	-	100	100	100

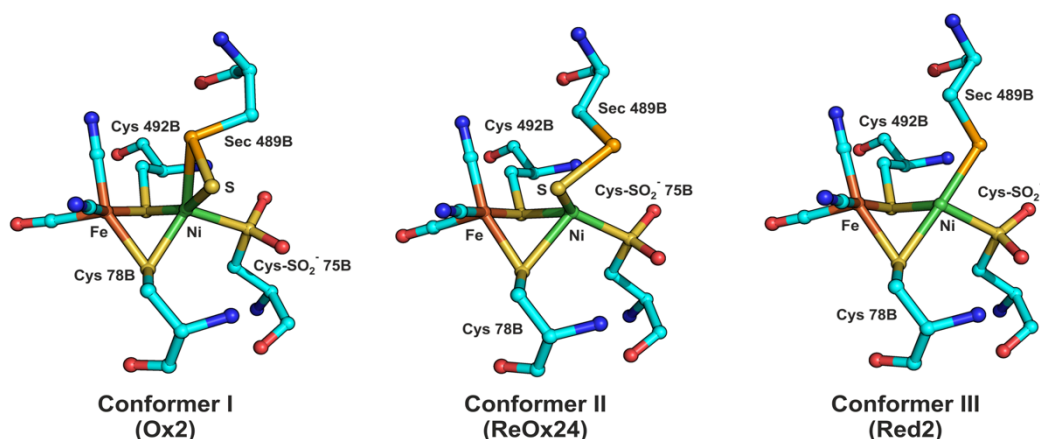


Figure 3.3. Overview of the active site structure of the active site in the different redox states of *DvH* NiFeSe Hase. The NiFe centre and the protein residues are drawn in ball-and-stick representation, with atom colours cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel, gold for selenium.

In both structures of the as-isolated oxidized forms Ox1 and Ox2, the three conformers of Sec489B are present, but with a predomination of conformer I (Figure 3.4). In the reduced Red1 structure the exogenous sulphur atom is no longer observed at the active site and the Sec489B residue is present in conformer III, in agreement with our earlier proposal that conformer III corresponds to the reduced state of the enzyme [37], by analogy with the crystal structure of the reduced *Db* enzyme [49]. In the Red1 structure the active site is present in this single conformation, whereas in the reduced Red2 structure (Figure 3.5), a small positive residual electron density region near conformer III of Sec489B was attributed to a slight contribution from conformer II (*ca.* 12%), included in the final PHENIX refinement, which may have been due to some partial re-oxidation during manipulation of the crystal.

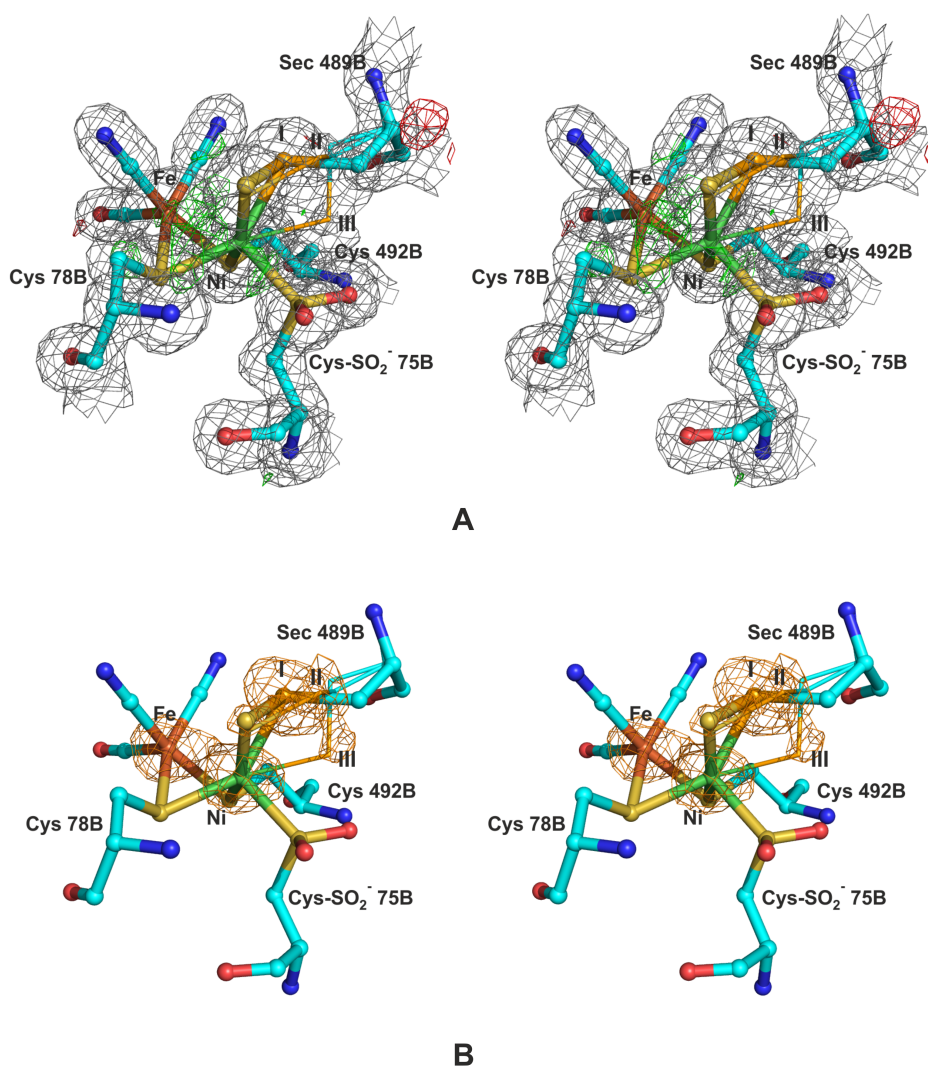


Figure 3.4 Stereoviews of the active site in the oxidized Ox2 structure of *DvH* NiFeSe Hase. The NiFe centre and the protein residues are drawn in ball-and-stick representation, with atom colours cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel, gold for selenium. The roman numerals denote the Se atom position in each of the three Sec conformers in this crystal structure and the two minor Sec conformers are shown as thin lines. (A) The final $2|F_o|-|F_c|$ map is drawn in gray at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green) and -3 (red) map r.m.s. level. (B) The final anomalous difference map is shown in orange

at the 4.5 map r.m.s. level.

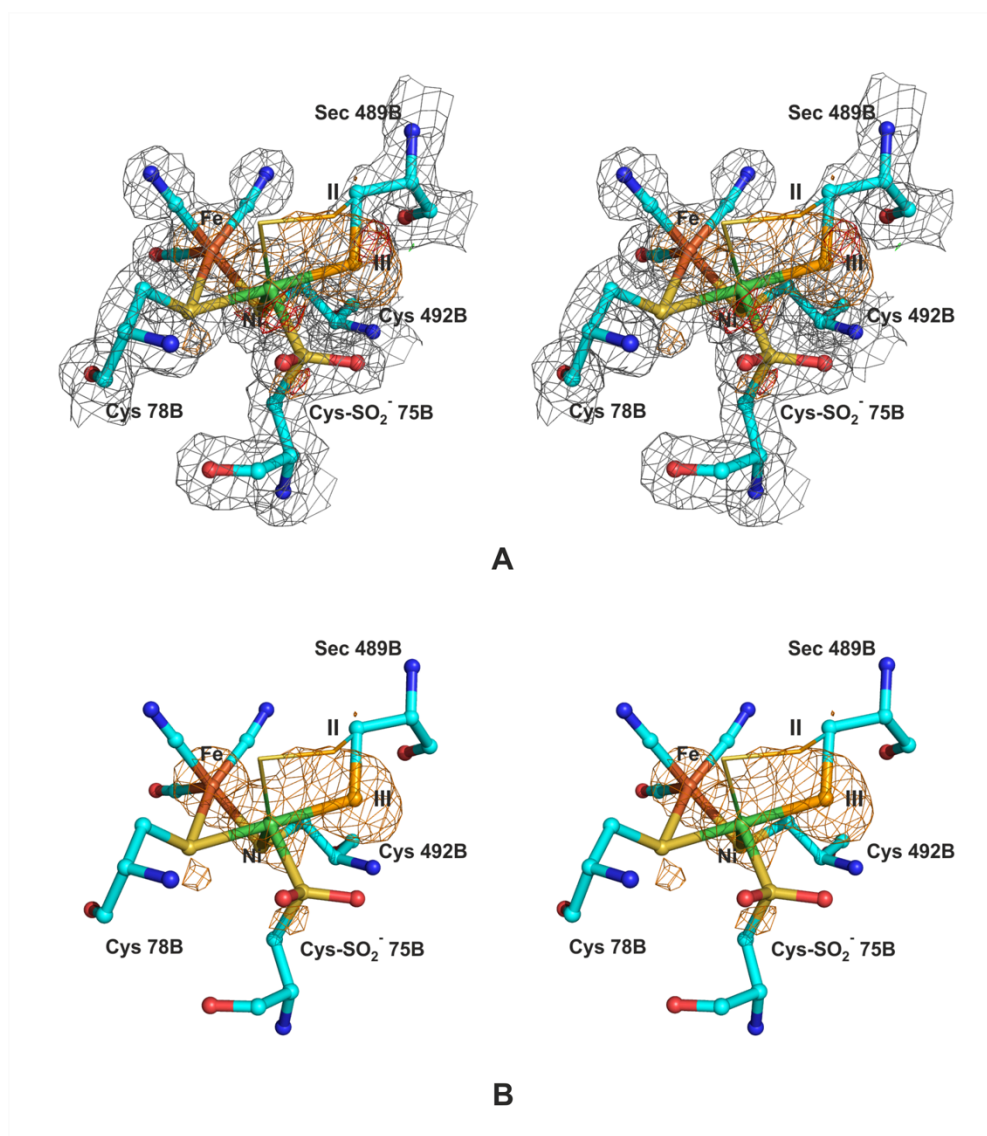


Figure 3.5 Stereoviews of the active site in the reduced Red2 structure of *DvH* NiFeSe Hase. The [NiFe] centre and the protein residues are drawn in ball-and-stick representation, with atom colours cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel, gold for selenium. The roman numerals denote the Se atom position in each of the two Sec conformers in this crystal structure and the minor Sec conformer present is shown as thin lines. (A) The final $2|Fo|-|Fc|$ map is drawn in gray at the 1.5 map r.m.s. level and the final $|Fo|-|Fc|$ map is represented at the 3 (green) and -3 (red) map r.m.s. level. (B) The final anomalous difference map is shown in orange at the 4.5 map r.m.s. level.

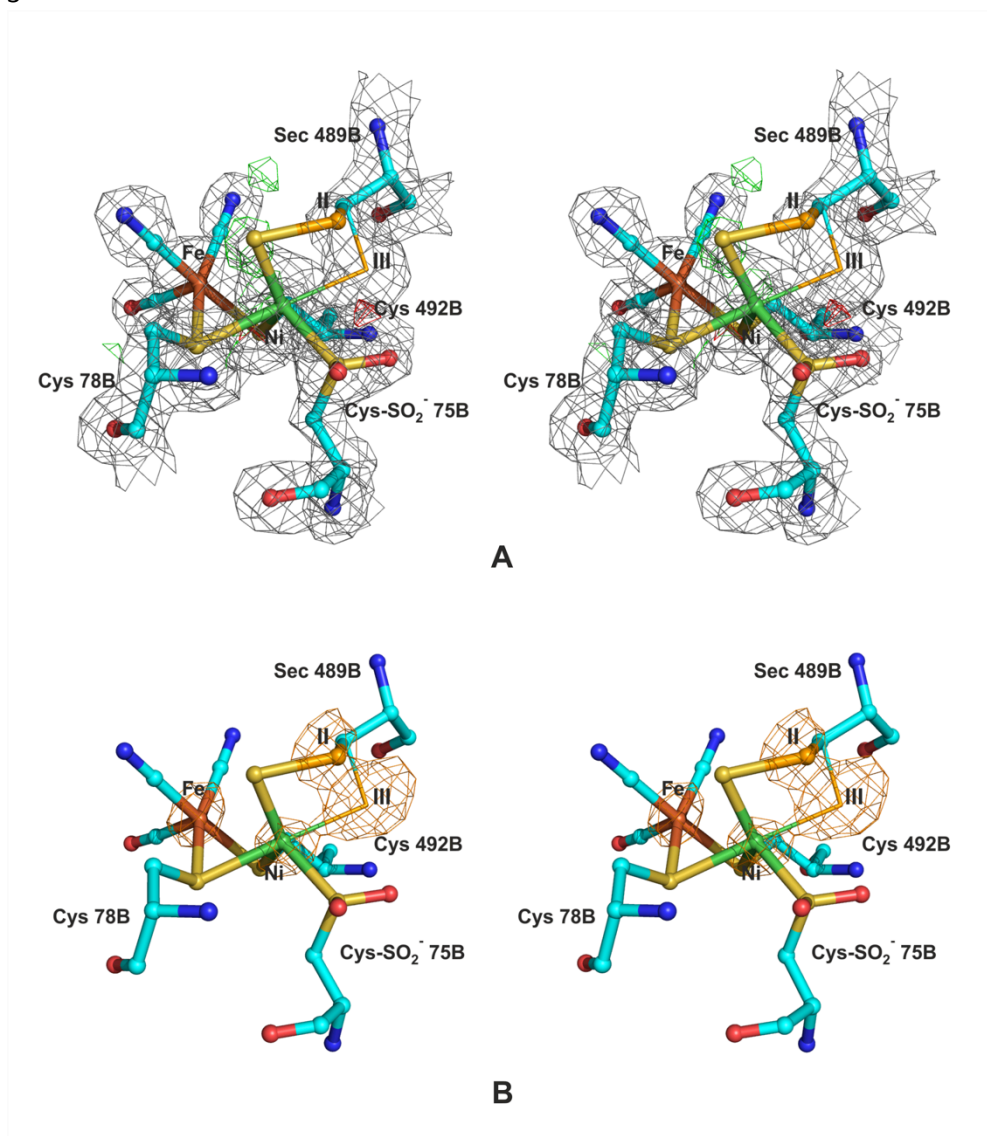


Figure 3.6 Stereoviews of the active site in the re-oxidized ReOx24 structure of *DvH* NiFeSe Hase. The NiFe centre and the protein residues are drawn in ball-and-stick representation, with atom colours cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel, gold for selenium. The roman numerals denote the Se atom position in each of the two Sec conformers in this crystal structure and the still dominant Sec conformer III is shown as thin lines. (A) The final $2|F_o|-|F_c|$ map is drawn in gray at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green) and -3 (red) map r.m.s. level.

Finally, in the re-oxidized structure ReOx24 (Figure 3.6) a higher percentage of molecules (38%) include the exogenous sulphur atom at the active site (conformer II), but conformer III is surprisingly still predominant (62% occupancy). No conformer I is present, which agrees with the FTIR results showing that the re-oxidized protein is in a different form from the as-isolated one [28]. After a 24h exposure to air the enzyme active site will most certainly be fully oxidized, therefore the observed predominance of conformer III suggests that the different conformers do not correlate directly with the oxidation state of the active site, but are rather related to the binding of the exogenous S atom. These observations can be rationalized by considering that reduction leads to displacement of this atom from the active site. Upon re-oxidation, the re-binding of this S atom is a slow process, justifying why 62% of the molecules in the re-oxidized state and ~10% in the oxidized as-isolated state are still found as conformer III, with no bound S. The conformer interconversion indicates that the S atom must remain bound to the protein and very likely occupies a position *ca.* 7.3 Å away from the Ni atom, in a solvent-accessible pocket, which in the Ox1, Ox2 and ReOx24 crystal structures is occupied by a Cl⁻ ion from the crystallization buffer. Although a proper anion transfer channel could not be identified in the crystal structures, the proposed coordination position for the sulphide ligand is located sufficiently close to the active site, such that protein dynamics due to thermal motion at room temperature should allow the displacement of the sulphide ligand between the active site and this other position. As previously noted [37] the three Sec489 conformers with similar relative occupancies were also observed in a crystal structure of the as-isolated oxidized form of DvH NiFeSe Hase obtained from 2.4 Å diffraction data measured *in house*. This rules out the possibility that the observation of

conformer III in the Ox1 and Ox2 crystal structures is an artefact caused by photoelectron reduction in the intense synchrotron X-ray beam.

The anomalous dispersion properties of the Se atom in Sec489B were enhanced by the X-ray wavelengths used in the data collections and required a modified refinement procedure. These properties were also helpful to confirm that the residual peaks observed around the main Sec489B conformation in the Ox1, Ox2 (Figure 3.4B), Red2 (Figure 3.5B) and ReOx24 (Figure 3.6B) crystal structures were indeed alternative positions for the Se atom, thus validating the presence of the other conformers (see Supplementary Results for details).

To our knowledge, the unusual double coordination of the Ni atom by a S and Se atoms has not been reported for any other protein structure, and a Cambridge Structural Database [50, 51] search for similar coordination motifs in small molecule compounds failed to give any results. However, there were several examples of double coordination of S-Se ligands to transition metals molybdenum, but only if the carbon atom bonded to Se was excluded from the search fragment (see Supplementary Results for details).

In the active site, several bond distances were refined using soft restraints, using large e.s.d. values (0.15 Å), to allow for a wider variation of the restrained bonds and ensuring a well-behaved refinement while avoiding the imposition of a fixed stereochemistry (see Supplementary Results). The mean Ni-Fe distance from the two oxidized structures is 2.42 Å and that from the two reduced structures is 2.50 Å, in agreement with the 2.53 Å observed in the reduced crystal structure of *Db* NiFeSe Hase [49]. Surprisingly, in the re-oxidized crystal structure, the Ni-Fe distance increased further to 2.68 Å. Higher-resolution crystal structures of the reduced and reoxidized forms of *DvH* NiFeSe Hase are needed to validate the significance of the observed variations, but

they suggest a behaviour of the Ni-Fe core in the active site that is different from that in O₂-sensitive and O₂-tolerant NiFe Hases: in these enzymes, the Ni-Fe distance decreases from 2.8-2.9 Å to *ca.* 2.5-2.6 Å when the enzyme loses its bridging ligand by reduction and increases back upon re-oxidation. In contrast, the Ni-Fe distance in *DvH* NiFeSe Hase increases slightly upon reduction and increases even further when the enzyme becomes reoxidized. This different behaviour may result from the conformational flexibility displayed by Sec489B (absent in NiFe Hases), combined with the chemical nature of the different ligands present in the oxidized state of the enzymes: a bridging oxygen species in O₂-sensitive and O₂-tolerant NiFe Hases and an exogenous sulphur atom bound to the Ni atom in *DvH* NiFeSe Hase.

To date it was not possible to obtain crystals after re-oxidation for periods longer than 24h, in order to investigate whether conformer II eventually converts back into conformer I, the predominant conformer observed in the oxidized, as-isolated form of the *DvH* NiFeSe Hase. Nevertheless, these results agree with the previous FTIR studies which showed that this Hase formed a state different from the as-isolated one upon reduction and re-oxidation [28], and reveal a remarkable structural complexity of the active site of this enzyme upon O₂ inactivation. It was previously shown that in O₂-sensitive NiFe-Hases oxidation may generate two different Ni (III) inactive states, Ni-A or Ni-B [32, 52]. This is in contrast with NiFeSe Hases from *DvH* and *Db*, which do not form Ni-A or Ni-B species even after reduction and exposure to oxygen [25, 49]. In agreement with this, the bridging oxide ligand between the Ni and the Fe is absent in the three redox forms of the *DvH* enzyme. It seems that the NiFeSe Hases form distinct unready states from the O₂-sensitive NiFe ones. This is also

reflected in the fact that the reactivation of these unready states is fast and requires a low redox potential [26, 28].

An oxidized cysteine is present in the active site

We previously reported that Cys75B, one of the terminal cysteines that bind the active site Ni atom, was found oxidized to cysteine sulphinic acid (Cys-SO₂H/Cys-SO₂⁻) in the structure of the as-isolated oxidized form of DvH NiFeSe Hase [37]. This residue modification is also present in the five crystal structures reported herein. Several crystal structures with oxidized cysteines have been described in the literature [53-56]. Oxidation of cysteine thiols to disulphides (-S-S-) or sulphenic acids (Cys-SOH) can be carried out by reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide or nitrogen dioxide [57]. This process is reversible, in contrast to oxidation of cysteines to sulphinic (-SO₂H) or sulfonic acids (-SO₃H), which is regarded as irreversible [57, 58]. However, in the case of peroxiredoxins formation of cysteine sulphinic acids was shown to be a reversible process *in vivo* [54]. In the present case, even though all the experiments to reduce the crystals were performed with dithionite and/or in an environment rich in H₂ and N₂, it is evident that the modified cysteine was not reduced, which supports the notion that this modification is irreversible. This process likely occurred during purification and/or crystallization, as cysteines can be selectively oxidized to cysteine sulphinic acid (Cys-SO₂H) and to cysteine sulfonic acid (Cys-SO₃H), even by air [53]. The successful reduction of Ox1 crystals (with a 100% oxidation of Cys75B to sulphinate) with a H₂/N₂ mixture to produce the crystal used in the Red1 structure determination suggests that some enzyme activity was still present in the crystals, since before reduction the crystals were transferred to a

fresh drop without free enzyme in solution. It has been suggested that in O₂-sensitive NiFe Hases oxidation of a terminal cysteine to sulphenate is related to the formation of the inactive Ni-A state (reviewed in [59]). However, while the O₂-sensitive NiFe Hases in the Ni-A state are inactive, our results suggest that the formation of a cysteine sulphinic acid in the NiFeSe Hase from *DvH* may not totally abolish enzyme activity. Nevertheless, and although the cysteine sulphinic acid seems to be present with 100% occupancy in all crystal structures so far determined for this enzyme, the presence of a small number of molecules with non-oxidized cysteines in the crystal cannot be ruled out. These molecules might then reduce the rest by electron transfer in the crystal. Further studies will be required to clarify this point.

In peroxiredoxins the biological relevance of cysteine-sulphinic acids has been well established, and understood as an alteration that results in protection against cellular oxidative stress [60]. In these cysteine-based peroxidases, the cysteine-sulphinic acid residues are exposed near the protein surface and can be reduced back to cysteines via a complex mechanism involving specific enzymes and requiring ATP [60]. In the *DvH* NiFeSe Hase, not only Cys75B is deeply buried inside the protein so that such a reduction process is unlikely, but no evidence exists in the genome of *DvH* for these cysteine-sulphinic acid-reducing enzymes.

There are no other known protein structures to date displaying a sulphinic acid coordination to Ni as found in the *DvH* NiFeSe Hase crystal structures reported in this work. However, several small molecular compounds with a similar Ni coordination could be found in the Cambridge Structural Database [50, 51] (see Supplementary Results for details). The study of these compounds has been stimulated by their role as models for the active site of

NiFe Hases, and the possibility that oxidation of the thiolate ligands to sulphenates or sulphinates could lead to enzyme inactivation has been long recognized (see for example [61]). Most of these compounds involve a diazo-dithiolate Ni coordination, but there are a few Ni-tetrathiolate molecules [62–64] where one or two sulphinate groups coordinate the Ni atom. Several diazo-dithiolate-Ni complexes with thiolate, sulphenate and sulphinate Ni ligands were studied electrochemically [61] and all show a reversible Ni(II)/Ni(I) redox transition. The oxidation scans of the sulphenate and sulphinate complexes showed irreversible transitions (not present in the scan of the sulphinate complex) that could correspond to the oxidation to sulphinate of the thiolate/sulphenate Ni ligands, and although a further feature was observed at even more positive potentials that might correspond to the Ni(II)/Ni(III) transition, it could not be validated chemically. A tetrathiolate-Ni compound with sulphinate Ni ligands was recently studied electrochemically and the results show only a quasi-reversible Ni(II)/Ni(III) redox transition [64]. Since the experiment only scanned positive potentials, the possibility of a Ni(II)/Ni(I) transition was not investigated. These results raise the possibility that in a NiFeSe Hase active site with a cysteine sulphinic acid ligand the Ni atom could still experience the same +1, +2 and +3 redox states as in a pristine active site. However, the transition potentials at which these transitions may occur could be sufficiently changed to cause the enzyme to be inactive.

The proximal iron–sulphur cluster

In O₂-sensitive NiFe and in NiFeSe Hases, the proximal [4Fe-4S] cluster is cubane-shaped and coordinated by four cysteine residues (Cys 17A, Cys 21A, Cys 115A and Cys 149A in *DvH*). In the previously reported structures of the

oxidized NiFe Hase from *Dd* ATCC 27774 (1.8 Å) [65] and the *DvH* NiFeSe Hase (2.04 Å) [37], the proximal iron-sulphur cluster was found in a partially oxidized form, identified as [4Fe-3S-3O] on the basis of its electron density. A modification of the proximal [4Fe-4S] cluster was also reported for the 3D structure of the membrane-bound O₂-sensitive NiFe Hase from *Av* in the oxidized Ni-A state [17]. However, the partial occupancy of the modified form combined with the 2.1 Å resolution of this structure did not allow a clear identification of the stereochemistry of the modified cluster. A similar oxidative modification has also been reported for a different type of Fe-S cluster in the crystal structure of the oxidized form of the Hybrid Cluster Protein from *DvH* [66].

In *DvH* NiFeSe Hase, the high resolution (1.5 Å) electron density maps of the as-isolated Ox1 crystal structure allowed for the first time a complete and detailed picture, revealing that in this case the cluster is super-oxidized to [4Fe-4S-3O] (Figure 3.7). Three of its iron atoms are bound to cysteine residues (Cys 17A, Cys 115A and Cys 149A) and the fourth is coordinated by three oxygen atoms and one of the carboxylate oxygens of Glu 77A (Figure 3.8A). This iron atom occupies a very similar position to that of Fe₂ in the modified proximal [4Fe-4S] cluster of the *Av* NiFe Hase, that in addition is coordinated by one of the carboxylate oxygens of Asp 75 in the small chain of the enzyme, structurally equivalent to the Glu 77 A in *DvH* (Figure 3.8B).

Instead of being released from the cluster as previously believed, the fourth sulphur atom of the cluster becomes attached to one of the oxygens and forms a persulphide bridge with Cys21A. In the Ox1 structure, the oxidation of the proximal [4Fe-4S] cluster is incomplete (*ca.* 80%), with a resulting overlap between the electron densities of both forms of this cluster, hindering the

elucidation of the stereochemistry of its oxidized form. In the as-isolated Ox2 crystal structure (1.33 Å resolution), the cluster was found in a *ca.* 100% oxidized form and confirmed our new interpretation of its chemical structure.

The identity of the newly identified sulphur atom was validated by its equivalent isotropic thermal motion parameter (17.2 Å²), very similar to those of Cys21A S^γ (17.8 Å²) and O1 (16.1 Å²).

Most stereochemical parameters for the oxidized cluster form were soft-restrained to ensure a well-behaved refinement, using larger σ values (0.05 Å and 5°) than usual to allow for a better accommodation of the atomic positions into the electron density. The stereochemistry of the super-oxidized part of the cluster (near atom Fe2, see Figure 3.8A and Supplementary Table 3.2) was analysed by comparison with similar parameters retrieved from the Cambridge Structural Database [50, 51] and with the exception of the O-S-S motif for which there are few examples, all stereochemical parameters were consistent with the statistical distributions of similar parameters in the CSD (see Supplementary Results for details).

It is very likely that the super-oxidation of the proximal cluster results from O₂ attack during the aerobic purification and/or crystallization of the enzyme. The fact that three oxygen atoms are present in the modified cluster suggests that a water molecule may also be involved. The proximal cluster is surrounded by three conserved water molecules, one of which (W57 in Figure 3.8A, W3128 in Figure 3.8B and W8 in Figure 3.8C) is located between the proximal cluster (*ca.* 4 Å) and the ordered solvent layer (*ca.* 6 Å). A water molecule has also been invoked in oxidation of the active site, as the source of the hydroxo ligand in the Ni-A/Ni-B species, with O₂ acting as the electron acceptor [32, 67]. The super-oxidation of the proximal cluster is not observed in

the Red1 (not shown) or Red2 (Figure 3.8C and Figure 3.9) structures of the enzyme, showing that it is reversed upon reduction. Furthermore, the fact that this modification is still not present in the ReOx24 structure (not shown) indicates that this reaction is a slow process that did not occur after 24h incubation at 20°C.

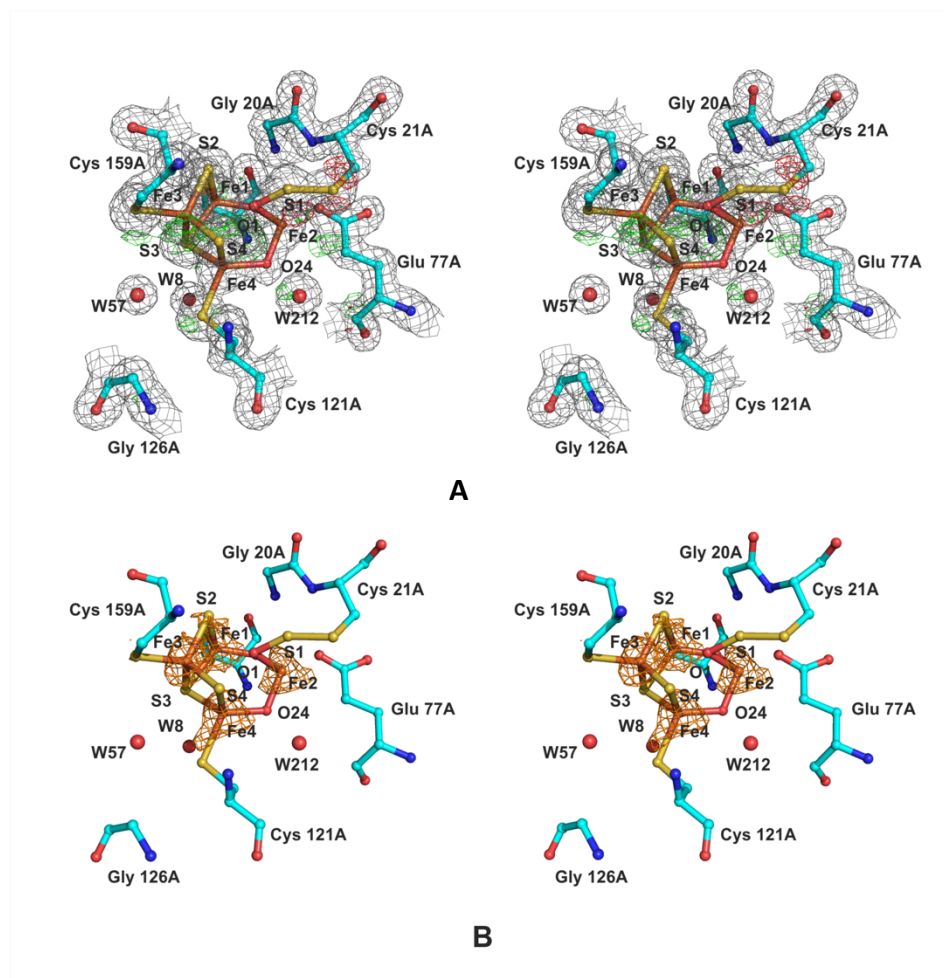


Figure 3.7. Stereoviews of the proximal [4Fe-4S-3O] cluster in the oxidized Ox2 structure of DvH NiFeSe Hase. The [Fe-S] cluster and the protein residues are drawn in ball-and-stick representation, and conserved water molecules are drawn as spheres. Atom colours are cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron; (A) The final $2|F_o|-|F_c|$ map is drawn in gray at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green) and -3 (red) map r.m.s. level. (B) The final anomalous difference map is shown in orange at the 4.5 map r.m.s. level. (B) The final anomalous difference map is shown in orange at the 4.5 map r.m.s. level

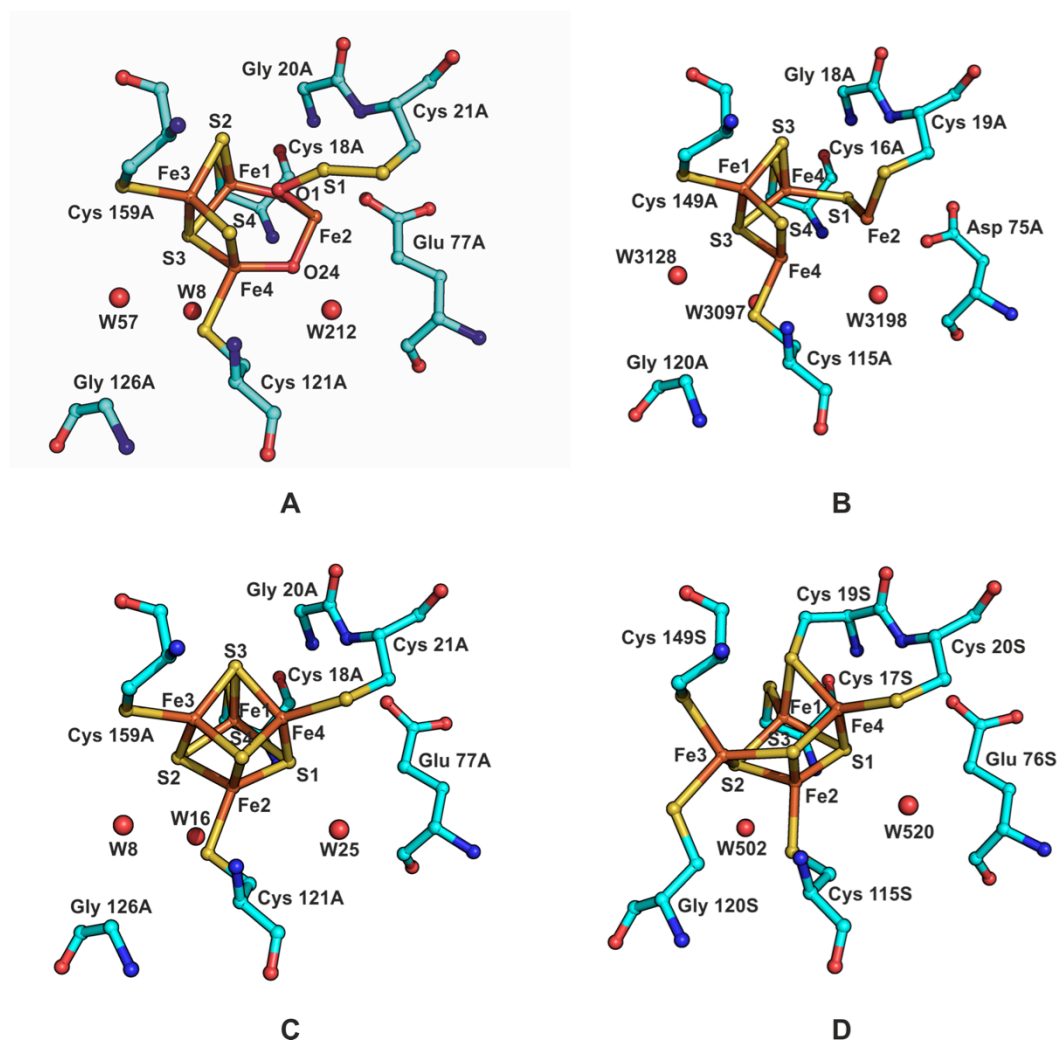


Figure 3.8. Overview of the proximal [Fe-S] cluster in structures of NiFeSe Hase from *DvH* and [NiFe] Hases from *Av* and *E. coli* (**A**) The [4Fe-4S-3O] form in the Ox2 crystal structure; (**B**) The distorted [Fe₄-S₄] in the crystal structure of the O₂-sensitive *Av* NiFe Hase in the Ni-A state (PDB 3myr); (**C**) The cubane [Fe₄-S₄] form in the Red2 crystal structure; (**D**) The [4Fe-3S] in the crystal structure of the O₂-tolerant *E. coli* NiFe Hase in the reduced state (PDB 3uqy). The [Fe-S] clusters and the protein residues are drawn in ball-and-stick representation, and conserved water molecules are drawn as spheres. Atom colours are cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron.

This is the first evidence that inactivation of the *DvH* NiFeSe Hase by O_2 involves the super-oxidation of the proximal [4Fe-4S] cluster, which may then act as a barrier against electron transfer with the active site, whereas in NiFe Hases the enzyme inactivation mainly results from the binding of an oxygen species at the bridging position of the active site. In addition, it also appears that full oxidation of the proximal cluster is not easily reversed by H_2 , since we could not obtain Red2 crystals from Ox2 crystals (where the proximal centre is ~100% oxidized to [4Fe-4S-3O]) by using only H_2 in a N_2/H_2 (85/15%) gas mix at ~1 atm, and in the absence of equipment for exposing the crystals to H_2 at high pressure as used by other authors [49], the Red2 crystals could only be obtained after addition of dithionite. This was not the case with the preparation of Red1 crystals from Ox1 crystals, where reduction (as indicated by blue colour of methyl viologen) was achieved only with H_2 , and thus it seems that a reasonable number of active molecules with intact clusters in the oxidized crystal (as in Ox1) are needed in order to supply electrons by intermolecular transfer to reduce the oxidized clusters of neighbouring molecules and restore their canonical composition and stereochemistry. In a crystal where very few if any intact clusters are present (as in Ox2), this process may be extremely slow or even impossible.

The recent crystallographic structures of O_2 -tolerant NiFe Hases from *Hm* (PDB 3ayx), *Re* (PDB 3rgw) and *Ec* (PDB 3uqy) showed that the proximal iron-sulphur cluster is of the form [4Fe-3S] and is coordinated by six cysteine residues [19, 22, 23] (Figure 3.8D). In O_2 -sensitive NiFe Hases and in NiFeSe Hases, where the proximal [4Fe-4S] cluster is coordinated by four cysteine residues, the two additional cysteines correspond to conserved glycine residues (Supplementary Figure 3.1). One of the sulphur atoms of the [4Fe-3S] cluster,

equivalent to S2 in the Ox1 and Ox2 structures (Figure 3.8A), and S3 in the Red1, Red2 and ReOx24 structures (Figure 3.8C), is replaced by S^γ from Cys 19 (3rgw and 3uqy) or Cys 25 (3ayx) in the small sub-units of the O₂-tolerant enzymes. The sixth cysteine residue is structurally equivalent to Gly 126A in DvH NiFeSe Hase and coordinates atom Fe3 in the proximal cluster (Figure 3.8C). As a result of these two structural changes, one of the Fe-S bonds is broken in the cluster and it assumes a distorted geometry in the O₂-tolerant enzymes. Several studies have pointed to this cluster as responsible for the O₂ tolerance displayed by this type of Hases [12, 18, 20, 21, 68], and it has been proposed that it can undergo two redox transitions instead of the single one that can take place in the canonical [4Fe-4S]^{2+/1+} cluster. This property allows reverse donation of two electrons to the active site, thus preventing the O₂-tolerant enzymes from reaching the unready Ni-A state [20]. Instead, their inactive state is always of the ready Ni-B form. It is also worth noting that in the crystal structures of the O₂-tolerant Hases one of the conserved water molecules near the proximal cluster (W57 in Ox2, see Figure 3.8A) is no longer present because of the steric hindrance due to the side chain of the sixth cysteine residue (Cys 120S in the O₂-tolerant *E. coli* NiFe Hase, PDB 3uqy, see Figure 3.8D). This observation raises the intriguing possibility that the role of this sixth cysteine, which has been established not to be relevant to the mechanism of O₂-tolerance involving the two redox states of the proximal [4Fe-3S] cluster [21], may be instead to protect this cluster from O₂ attack in a process similar to that resulting in a [4Fe-4S-3O] proximal cluster as observed in the Ox1 and Ox2 structures of the DvH NiFeSe.

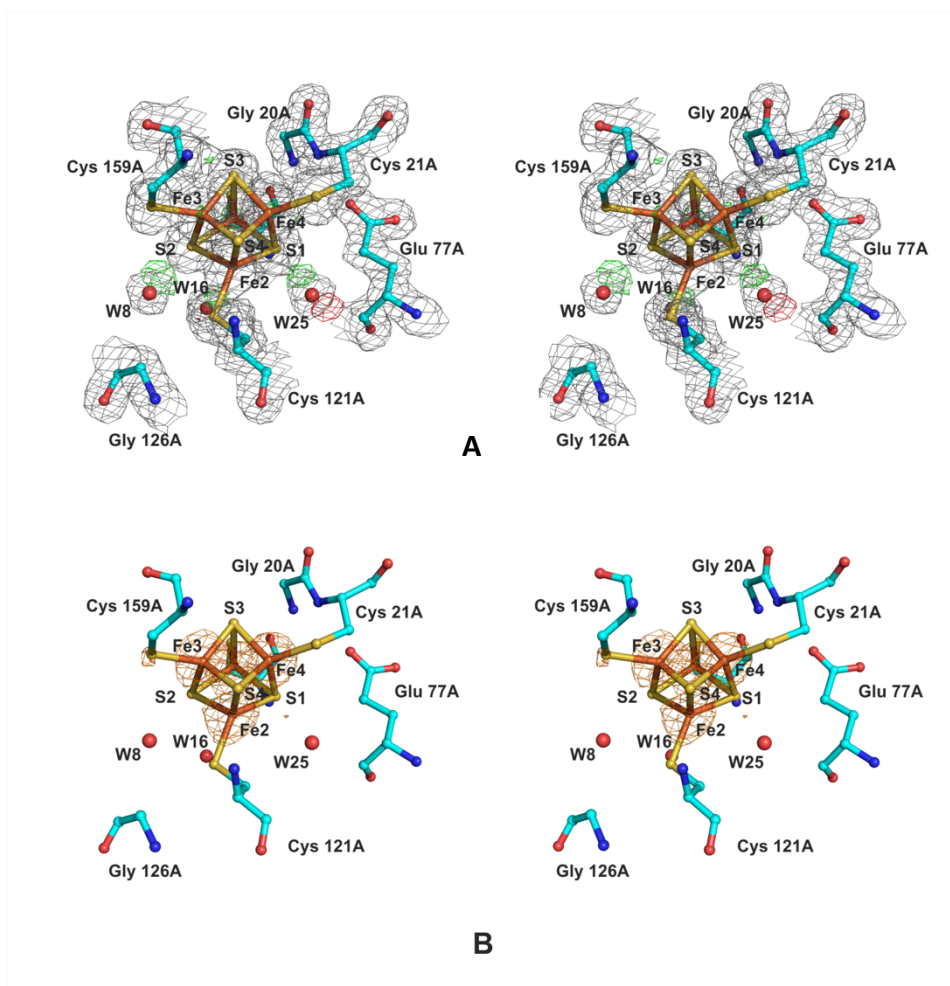


Figure 3.9. Stereoviews of the proximal [4Fe-4S] cluster in the reduced Red2 structure of *DvH* NiFeSe Hase. The [Fe-S] cluster and the protein residues are drawn in ball-and-stick representation, and conserved water molecules are drawn as spheres. Atom colours are cyan for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron; (A) The final $2|F_o|-|F_c|$ map is drawn in grey at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green) and -3 (red) map r.m.s. level. (B) The final anomalous difference map is shown in orange at the 4.5 map r.m.s. level.

3.5 CONCLUSIONS

The crystal structure of the soluble form of the NiFeSe Hase from the sulphate-reducing bacterium *DvH* was determined in three redox forms: as-isolated oxidized (Ox1 and Ox2), reduced (Red1 and Red2) and re-oxidized (ReOx24). Both oxidized crystal structures were determined to higher resolution (1.5 Å and 1.33 Å, respectively) than the previously reported crystal structure of the same form (2.04 Å) [37], thus providing a more detailed structural picture of the oxidized form of this enzyme, which was essential to elucidate the structure of the modified proximal cluster. The 3D structure of the *DvH* NiFeSe Hase is largely identical to that of a classic NiFe Hase, but there are important differences around the NiFe binuclear active site. In the structures of the as-isolated oxidized forms Ox1 and Ox2, the Se atom in Sec489B is present in three conformations, termed conformers I, II and III, with conformer I being predominant. These conformers correspond to the three different rotamers of Sec489B (defined by the dihedral angles about the C^α-C^β bond). The structures of the other two redox states reveal a geometry of the active site where the Sec489B is in similar conformations to conformers II and III. In the Red1 structure only conformer III is detected, while in the Red2 structure a small conformer II component is also observed. Conformer III corresponds to the only conformer observed for the equivalent cysteine residue in NiFe Hases and is also observed in the crystal structure of the NiFeSe Hase from *Db* in the reduced state [49]. In the crystal structure of the re-oxidized enzyme (ReOx24), conformers II and III of Sec489B are present, with a predomination of conformer III. In conformers I and II an exogenous sulphur atom binds the Se atom of Sec489B (and also the Ni atom in the case of conformer I). It appears

that it is the binding of this S atom, rather than the oxidation state that determines which conformer is present. This binding is apparently a slow process compared to the reduction/re-oxidation of the protein, resulting in a mixture of conformers in different oxidation states. The consequence of the conformational changes of Sec489B in comparison to the corresponding Cys residue in NiFe Hases is that the Se atom blocks access to the bridging position of the active site of small molecular species such as O₂, and thus may prevent the formation of a bridging oxo- ligand, usually implicated in the Ni-A and Ni-B electronic states of NiFe Hases [13, 69]. However, recent experiments have shown that the Ni-A/Ni-B states can also be reached in the absence of O₂, but in the presence of strong oxidizing agents or a high redox potential, which possibly generate reactive oxygen species close to the active site [32]. Also, in contrast with O₂-sensitive and O₂-tolerant NiFe Hases, the Ni-Fe distance in the NiFeSe Hase increases slightly upon reduction of the as-isolated oxidized form, a larger increase being observed when the reduced form is allowed to reoxidize in air. An additional feature observed at the active site of the DvH NiFeSe Hase is the oxidation of the terminal cysteine bound to the Ni atom to cysteine sulphinic acid/sulphinic acid. This modification is not reversible by reduction of the enzyme under the conditions used.

Finally, the high-resolution structures of the oxidized state enabled the structural and stereochemical elucidation of the modification of the proximal-iron sulphur cluster that was found oxidized to a [4Fe-4S-3O] form. A similar form of this cluster was previously reported for the three-dimensional structure of the O₂-sensitive NiFe Hase from *Dd* ATCC 27774 [65], and may result from O₂ attack involving also a water molecule, during aerobic purification and/or

crystallization. The higher resolution (1.5 Å and 1.33 Å respectively) of the Ox1 and Ox2 crystal structures allowed the clarification of the chemical composition of the modified cluster, showing that one of the sulphur atoms was not lost as previously believed [37, 65], but instead formed a persulphide bridge with a cysteine coordinating the unmodified cluster. Three water molecules around the proximal iron-sulphur cluster are conserved in the known 3D structures of O₂-sensitive NiFe Hases, revealing a certain degree of solvent exposure of this cluster, even though it is buried inside the protein, whereas in the known 3D structures of O₂-tolerant NiFe Hases one of these three molecules is displaced by one of the extra Cys residues coordinating the proximal iron-sulphur cluster. In the oxidized crystal structure Ox2 the proximal iron-sulphur cluster was almost completely oxidized to [4Fe-4S-3O]. Contrary to our experience with the Ox1 sample where the proximal iron-sulphur cluster was ca. 80% oxidized to [4Fe-4S-3O], crystal reduction of Ox2 with a mixture of H₂/N₂ at 1 atm was not possible. Thus, it is becoming increasingly apparent that oxidation of the proximal iron-sulphur cluster may be an important modification in the inactivation of some O₂-sensitive NiFe and NiFeSe Hases. In fact, it is still not clear what the difference between the Ni-A and Ni-B inactivated states is, as the hydroperoxo bridging ligand believed to correspond to the Ni-A state of *Df* NiFe Hase (PDB 1yqw) [69] is not present in the *Av* NiFe Hase in the same state (PDB 3myr) [17]. The proposal for a hydroperoxo ligand stemmed from the fact that an elongated electron density was observed at the bridging position [69]. The high-resolution (1.04 Å) crystal structure of the NiFe Hase from *DvMF* in the Ni-A state (PDB 1wui) [56] also displayed a diatomic oxygen species at the bridging position of the NiFe active site, along with the partial oxidation to sulphenate of one of the bridging cysteines and of the terminal cysteine coordinating the Ni

atom that corresponds to Sec489B in the NiFeSe Hase from *DvH*. However, it is also conceivable that the electron density assigned to a diatomic oxygen species in both Ni-A structures may actually represent two different positions of an hydroxo ligand: one in a bridging position between the Ni and Fe atoms, and a second where the oxo-species only coordinates the Ni atom with a coordination distance of *ca.* 2 Å.

The Ni-A and Ni-B states have very similar spectroscopic properties (namely in FT-IR that is extremely sensitive to the active site electronic state), pointing to similar configurations of the active site. Indeed, our results suggest that the difference between the two states might also involve in some cases the oxidation state of the proximal cluster, as similar modifications seem to be present in the crystal structures of the O₂-sensitive Hases from *Av* [17] and *Dd* ATCC 27774 [65]. Thus, the Ni-A state in O₂-sensitive Hases could arise from different structural features at or near the active site in different enzymes, rather than a single one as usually believed. In addition, it seems that different enzymes may form different oxidized species. In this context, it should be noted that very different protocols can be used to originate Ni-A species, such as the case of the *Av* NiFe Hase that was aerobically purified already mainly in the Ni-A state [17], whereas the Ni-A state of the *DvMF* NiFe Hase was obtained by treatment with Na₂S [56]. The small changes in FTIR frequencies and EPR *g*-values between O₂-sensitive NiFe Hases in the Ni-A and Ni-B states may be rationalized in terms of small structural changes closer to the Ni atom or larger structural changes further away. The bridging ligand is typically *ca.* 2 Å from the Ni atom whereas the centre of the proximal Fe-S cluster is *ca.* 13 Å distant from the Ni atom. However, in the case of the *Av* NiFe Hase there is no evident structural change at or near the active site other than the oxidized proximal Fe-

S that can account for the Ni-A state of the enzyme in the crystal. Furthermore, a more distant interaction of Ni^{3+} with the medial $[\text{3Fe-4S}]$ cluster has been implicated in the complex splitting of EPR g -signals from some NiFe Hases, possibly mediated by an extra Fe^{3+} species located close to or in a modified proximal Fe-S cluster (see [17] and references therein).

The understanding of the structural factors behind the different behaviour displayed by NiFeSe Hases in comparison with O_2 -sensitive NiFe Hases has become a subject of our research, since NiFeSe Hases can be used in biotechnological applications, as shown by the performance of *Db* NiFeSe Hase in experimental H_2 -producing devices [10, 29] and the direct electron transfer observed after immobilization of *DvH NiFeSe* Hase in gold electrodes [27, 30].

The crystals structures reported herein provide important insights into how NiFeSe Hases are inactivated by O_2 . A main achievement is the elucidation of the structure of the modified proximal FeS cluster upon inactivation, which seems to involve reaction with O_2 and also a water molecule. Our results show that this modification is a slow process that is reversible upon reduction, in contrast to the formation of a cysteine-sulphinic acid at the active site, which is still present in the reduced form of the enzyme. Further detailed time-resolved studies of this enzyme are planned to evaluate the relative timing of these two oxidative processes, and their relative impact on the catalytic activity of the enzyme. These studies will be essential for the future design of engineered proteins with enhanced oxygen tolerance and catalytic properties to be used in bio-hydrogen production or hydrogen-based bioelectrical devices.

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3.4. Supplementary Results

The proximal iron–sulphur cluster

The stereochemistry of the oxidized part of the cluster (near atom Fe2, see Figure 3.3A and Supplementary Table 3.2) was analysed by comparison with similar parameters retrieved from the Cambridge Structural Database (CSD) v. 5.33 updated up to February 2012 [1, 2]. Since there are no exact matches for this metal cluster, three motifs were considered: Fe1-O-Fe2 (as in Fe2-O12-Fe1 and Fe2-O24-Fe4), Fe-O-S (as in Fe2-O1-S1) and O-S1-S2 (as in O1-S1-S^c Cys 21A). A restraint of two bonded atoms was added for the central O atom in the first two motifs and for all the atoms in the third. Statistics for these motifs are presented in Supplementary Table 3.3 and histograms calculated from the bond distances and angles retrieved for the Fe1-O-Fe2 and Fe-O-S motifs are included in Supplementary Figure 3.2). Motif O-S1-S2 had very few representatives in the CSD and no histograms could be calculated. The values derived from the Ox2 structure are not inconsistent with the range of values obtained from the small-molecule examples. However, the Fe2-O1 bond (1.93 Å) and the Fe2-O1-S1 angle (83°) are at the lowest limits and the O1-S1 bond (1.67 Å) is at the higher limit of the value ranges found for this motif (Fe-O-S). Nevertheless, the mean value of the same O1-S1 bond in the O-S-S motif (1.66±0.01 Å) is very close to its refined value in the Ox2 structure and in addition the S1-S^c Cys 21A (1.90 Å) is also reasonably close to the mean value of the S-S bond (1.96±0.01 Å) in this motif. The O-S-S angle (154°) is much larger than that found in the small-molecule structures 108.2±4°, but the number of examples (10) is rather small and possibly not representative.

The structure of the active site in the different redox forms

The presence of a selenium atom in Sec489B combined with the choice of a short wavelength near its absorption edge K for the diffraction data measurements required the inclusion of the anomalous dispersion parameters for Se in the refinement, in order to avoid larger refined thermal displacement parameters for this atom as well as significant negative features in the residual electron density maps. Near the absorption edge, in addition to a significant anomalous dispersion contribution to the atomic scattering factor of Se ($f'' \sim 3.8$ e-) there is also a non-negligible dispersive component ($f' \sim 4$ e-), which causes the Se atom to scatter X-rays as if it were a lighter atom ($Z' \approx Z - 4$). The anomalous dispersion properties of the Se atoms enhanced by this choice of wavelengths were also helpful to confirm that the residual peaks observed around the main Sec489B conformation in the Ox1, Ox2, Red2 and ReOx24 crystal structures were indeed alternative positions for the Se atom, thus validating the presence of the other conformers.

Selected bond distances in the active site and refined occupancy factors for the three conformers are presented in Supplementary Table 3.4. To ensure a well-behaved refinement while avoiding the imposition of a fixed stereochemistry, these distances were refined using soft restraints, with large e.s.d. values (0.15 Å), allowing for a wider variation of the restrained bonds. The refined values for the Ni-S and S-Se bond distances are consistent for the three conformers with higher occupancies (conformer I in Ox1 and Ox2, conformer II in ReOx24) whereas for the three conformers with lower occupancies (conformer II in Ox1, Ox2 and Red2) those values show a variation that is probably due to the ambiguity introduced by the near-overlapped positions of the sulphur atom in conformers I and II. The refined Ni-Se bond distance is also

consistent throughout the five crystal structures, with the notable exception of ReOx24, where it is largest. However, and on the basis of the lowest estimate of the mean coordinate error (Cruickshank DPI in Table 3.2) the difference between the two extreme values is only 1.2σ , therefore probably not significant. The Ni-Fe distance varies between 2.42 Å in the Ox1 structure and 2.68 Å in the ReOx24 structure. This difference corresponds to 2.9σ , which is bordering on significant at the 99% confidence level. However, because Se, Ni and Fe atoms have higher atomic numbers it is reasonable to expect that their atomic coordinates are more accurately determined than those of the non-metal lighter atoms of the enzyme. In consequence, their mean coordinate errors are likely overestimated by the values listed in Table 3.2, as are the above error estimations of the stereochemical parameters involving these heavier metal atoms. In this case, the existence of several replicates of the same or similar parameters can help decide whether the observed differences are significant. The Ni-Se bond distances for the two oxidized (Ox1 and Ox2) crystal structures are very similar (mean value of 2.34 Å) but the values for the two reduced structures show a wider scatter with a mean value of 2.32 Å, raising doubts as to whether the larger value of 2.46 Å observed for the ReOx24 structure represents a significant change. On the other hand, the Ni-Fe distances from the two oxidized (mean value of 2.42 Å) and the two reduced (mean value of 2.50 Å) structures are consistent, despite the lower resolution (1.95 Å) and data quality of the Red1 crystal (see Table 3.1). The Ni-Fe bond distance values in the two reduced structures are also in agreement with that of 2.53 Å observed in the reduced crystal structure of *Db* NiFeSe Hase at 2.15 Å resolution [3].

Since there are no other known protein structures displaying the Ni coordination found in the *DvH* NiFeSe Hase crystal structures reported in this work, the stereochemistry of the several unusual structural motifs involved in the coordination of the Ni atom was also analysed by comparison with geometrical parameters retrieved from the CSD [1,2] and the results are presented in Supplementary Table 3.4. The Ni-SO₂-C motif was selected to represent the cysteine sulphinic acid bonded to the Ni atom, and 28 small molecule structures containing this motif were retrieved. The C-Se(M)S motif was chosen to emulate the dual coordination of the Ni atom by S and Se atoms in conformer I as displayed in Figure 3.3A. Since this search produced no hits, the search fragment was relaxed to include only S-Se dual coordination to any transition metal atom, and this search now revealed 8 small molecules containing 44 copies of the search motif, with the S-Se fragment mainly bound to molybdenum atoms, although a few examples containing iron and tungsten were also found. Finally, a search was performed against the motif C-Se-S-M, representing the Ni coordination found in conformer II as illustrated in Figure 3.3B, and only 2 structures were found, containing 3 examples of the search motif, with the S atom bound to either a Ni or a tin atom. The bond distances between the Ni, S and Se atoms are included in Supplementary Table 3.5 and are in agreement with those obtained from the refinement of the *DvH* NiFeSe Hase crystal structures listed in Supplementary Table 4. The S=O distances from Supplementary Table 3.4 were used to set the S=O^{b1} and S=O^{b2} bond distance restraints in the dictionary for the cysteine sulphinic acid residue that was used in the crystal structure refinements.

Supplementary Table 3.1 . SSM superposition of the different Hase structures

Moving	Ox2		Red1		Red2		ReOx24	
Fixed	Chain A	Chain B	Chain A	Chain B	Chain A	Chain B	Chain A	Chain B
Ox1	0.75 (276)	0.24 (481)	0.74 (276)	0.15 (481)	0.77 (276)	0.23 (481)	0.15 (277)	0.09 (481)
Ox2	--	--	0.28 (277)	0.23 (481)	0.20 (279)	0.16 (481)	0.78 (276)	0.25 (481)
Red1	--	--	--	--	0.28 (277)	0.18 (481)	0.76 (276)	0.12 (481)
Red2	--	--	--	--	--	--	0.77 (276)	0.21 (481)

The first number is the root-mean-squared deviation (Å) between the number of aligned C α atoms (in parenthesis).

Supplementary Table 3.2. Selected geometrical parameters for the oxidized proximal [4Fe-4S] cluster in the Ox2 crystal structure

	Refined value (Å)	Restrained value (Å)
Bond distance		
Fe1-O12	1.85	1.90 (5)
Fe2-O1	1.95	2.10 (5)
Fe2-O12	1.79	1.90 (5)
Fe2-O24	1.84	1.90 (5)
Fe2-O ² Glu 77A	2.08	2.05 (15)
Fe4-O24	1.92	1.90 (5)
S1-O1	1.67	2.15 (15)
S1-S Cys 21A	1.91	2.02 (10)
	Refined value (°)	Restrained value (°)
Bond angle		
Fe2-O1-S1	83	n/a
Fe2-O12-Fe1	119	115 (5)
Fe2-O24-Fe4	115	120 (5)
O1-S1-S Cys 21A	154	n/a

Supplementary Table 3.3. Cambridge Structural Database analysis of selected geometrical parameters in the oxidized proximal [4Fe-4S] cluster of the Ox2 crystal structure.

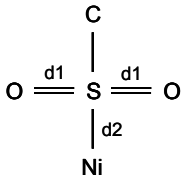
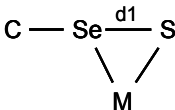
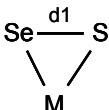
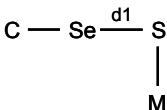
Motif A-B-C	Nr. structures (nr. motifs)	distance A-B (Å) mean (e.s.d.)	distance B-C (Å) mean (e.s.d.)	angle A-B-C (°) mean (e.s.d.)
Fe1-O-Fe2	647 (737)	1.80 (5)	1.79 (5)	150 (24)
Fe-O-S	195 (416)	2.07 (8)	1.48 (3)	136 (12)
O-S1-S2	5 (10)	1.48 (3)	1.96 (1)	108.2 (4)

Supplementary Table 3.4. Selected geometrical parameters for the active site in the refined DvH NiFeSe Hase crystal structures.

Structure	Ox1			Ox2			Red1	Red2		ReOx24		
Data resolution (Å)	1.50			1.33			1.95	1.82		1.80		Restraint value, Å
Conformer	I	II	III	I	II	III	III	II	III	II	III	
Distance Ni-Se, Å	2.35	-	2.43	2.34	-	2.32	2.27	-	2.36	-	2.42	2.39 (15)
Distance Ni-S, Å	2.13	2.09	-	2.16	1.89	-	-	2.55	-	2.12	-	2.20 (15)
Distance S-Se, Å	2.21	2.07	-	2.17	2.15	-	-	2.05	-	2.26	-	2.15 (15)
Distance Ni-Fe, Å	2.42	-	-	2.43	-	-	2.50	-	2.51	-	2.68	2.50 (15)

The data in boldface refer to the conformer with the highest occupation factor in each of the five structures, taken as a representative of that conformer.

Supplementary Table 3.5. Cambridge Structural Database analysis of selected structural motifs in the active site of the *DvH* NiFeSe Hase.

Motif	Nr. structures (nr. motifs)	distance d1 (Å) mean (e.s.d.) in CSD range in CSD	distance d2 (Å) mean (e.s.d.) in CSD range in CSD
	28 (34)	1.46(2) 1.35 - 1.55	2.13 (2) 2.05 - 2.19
	0	n/d	n/a
	8 (44)	2.20(5) 2.16 - 2.39	n/a
M = Mo, Fe, W			
	2 (3)	2.21(1) 2.20 - 2.26	n/a
M = Sn, Ni			

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CHAPTER 4

Homologous expression, biochemical and structural studies of the soluble *DvH* NiFeSe Hase and its engineered NiFe homologue

Raquel Ramos and Kimberly Keller have constructed the expression vector pMOIP3 and the IPAR01 strain, respectively. Oscar Gutierréz-Sanz and Dr. Antonio de Lacey have performed the FTIR spectra, electrochemistry and mass-spectrometry experiments and Marta C. Marques has performed the remaining experimental work. Manuscript in preparation.

ABSTRACT

In this chapter the development of a homologous system for recombinant expression of a NiFeSe Hase in *DvH* is described. This expression system enabled the production of a mutant variant, in which the Sec residue (U) was replaced by a cysteine (C). The resulting enzyme (U489C Hase) represents a NiFe Hase equivalent of the NiFeSe Hase. A complete characterization of the recombinant NiFeSe Hase and its sulphur analogue U489C Hase was carried out, and four high-resolution X-ray crystallographic 3D structures are presented, that provide further insights into the O₂ inactivation processes in this group of enzymes. Surprisingly, the first X-ray crystallographic structure of the U489C Hase revealed a Ni-depleted enzyme. We show for the first time that it is possible to (at least partly) reconstitute *in vitro* a NiFe Hase, which allowed for detailed functional studies and a spectroscopic characterization of the enzyme. Based on these results we propose new O₂-inactivation mechanisms for the NiFeSe Hases. This study aims to contribute to the design of improved biocatalysts for H₂ production.

4.1 INTRODUCTION

As previously mentioned (chapter 1, section 1.6), in NiFeSe Hases, one of the Ni Cys ligands is replaced by a Sec [1–3]. In contrast to standard NiFe Hases, the aerobically isolated selenoenzymes are easily activated, display higher catalytic activities and H/D exchange rates, decreased oxygen sensitivity and absence of EPR-active, oxidized states [3, 4]. To date, two NiFeSe Hases from *Db* and *DvH* have been crystallized and the overall structural fold was shown to be similar to group 1 NiFe Hases. In the active site of the first *DvH* NiFeSe Hase X-ray crystallographic structure [2] an S atom bound to the Ni atom was found with partial occupation and the side chain of the Sec residue was present in three conformations. This novel double coordination of the Ni atom by S and Se atoms had not been reported for any other protein structure at the time [5]. The terminal cysteine (C75 in the large subunit) was found modified to sulphinate, a modification that is reported as irreversible [6, 7]. As described in chapter 3, the crystal structures of the *DvH* NiFeSe Hase in different redox states at near-atomic resolution allowed a more detailed picture of the changes occurring at the proximal [4Fe-4S] cluster, revealing that in the oxidized states this cluster is in a super-oxidized [4Fe-4S-3O] form instead of [4Fe-3S-3O] as previously proposed [2, 5]. In contrast, recent crystal structures of the *Db* NiFeSe Hase indicate that oxygen exposure converts the Sec into an oxidized seleninate [8], never observed in the *DvH* oxidized structures. Volbeda and co-workers proposed that due to the high nucleophilic character of a selenate the resulting oxides (selenic acid and seleninic acid) can be rapidly reduced in the presence of a reducing agent, and thus that the Sec-enzyme can be easily reactivated [8].

The different structural data from these two NiFeSe Hases emphasize the fact that distinct NiFeSe Hases can form different oxidized states. Furthermore, the redox states observed in the 3D structures are also strongly dependent on the purification protocol. Although NiFeSe Hases were regarded in the past as O₂-resistant, the recent published results [5, 8] enabled the identification of unique inactivation pathways, distinct from those observed for NiFe Hases. The possibility of using O₂-resistant Hases in biotechnological devices stimulates current research on their inactivation mechanisms.

To investigate the structural basis of the *DvH* NiFeSe Hase inactivation processes, a homologous expression system for the soluble *DvH* NiFeSe enzyme was designed. This allowed the production of engineered NiFeSe Hases by site-directed mutagenesis. This system was used to generate a mutated protein where the Sec residue was replaced by Cys, thus converting the NiFeSe active site into a NiFe active site. Surprisingly, this single (and apparently simple) mutation had a strong impact in the assembly of the active site, resulting in a Ni-depleted form of the enzyme, very sensitive to O₂ attack.

In this work the kinetic and spectroscopic characterization of the recombinant NiFeSe Hase and its sulphur analogue U489C Hase are described. In addition, four high-resolution 3D structures, obtained by X-ray crystallography are presented. The recombinant Hases were purified both aerobically and (semi)-anaerobically. To reconstitute the Ni-depleted U489C a new assay was developed, resulting in the first experimental evidence that it is possible to rebuild the active site of a Ni-depleted Hase and recover (at least in part) its activity, independently of the presence of maturation proteins. The results obtained demonstrate that substitution of the Sec residue by Cys can greatly affect the inactivation processes of *DvH* Hases by O₂, resulting in a highly

oxidized enzyme with very low catalytic activity, and highlighting the role of the selenocysteine in affording a measure of protection against O₂ attack.

4.2 MATERIAL AND METHODS

4.2.1 Genetic constructions

A mutant strain lacking the *hysAB* genes of the NiFeSe Hase was produced by double homologous recombination in *DvH* according to [9], generating strain IPAR01 (electroporation parameters: 1500V, 250 Ω, and 25 μF). The pMOIP01 plasmid for the *hysAB* deletion was obtained by sequence ligation independent cloning (SLIC) [10]. Three segments were amplified by PCR: 1,055 bp upstream of *hysAB* (*hysAB* Up Fw – GCCTTTTGCTGGCCTTTTGCTCACATGGACAAGGATGAGCCCGTTGTGAA and *hysAB* Up Rev – AAGACTGTAGCCGTACCTCGAATCTATGCAGTGCCAGCCAATAGAGTGAA), 885 bp downstream of *hysAB* (*HysAB* Dwn Fw – AATCCGCTCACTAAGTTCATAGACCGGACGCCCATGATGTTAGGGTTCCAA and *HysAB* Dwn Rev – CGAGGCATTTCTGTCCTGGCTGGGCGTACGCATTACGCACGTATCAT) and the kanamycin resistance gene from pSC27 [9] (Kan 1 Fw – TAGATTCGAGGTACGGCTACAGTCTTACGGTCACAAACAGGTACGCCCCAGAGTC and Kan 2 Rev CGGTCTATGAACTTAGTGAGCGGATTCTCGTGTAGCCGATGCAGTGAGGTAGCTTGACAG); and then added to the pMO719 background via SLIC. Products from the amplifications were transformed into *E. coli* α-select Silver Efficiency (Bioline®) and successful transformants were isolated on LB medium containing 50 μg/ml of Kanamycin and 100 μg/ml of Spectinomycin. Correct isolates were identified by the expected PCR amplicons from the plasmids

constructs and also by sequencing performed at the DNA Core Facility at the University of Missouri, USA. The pMOIP01 plasmid was electroporated into *DvH* according to [9, 11], from which strain IPAR01 was obtained, by selecting with MOYLS4 medium containing 400 µg/ml of geneticin. The deletion of *hysAB* was confirmed by Southern blot using as probe an upstream fragment of the *hysAB* genes. A strategy to produce the soluble form of HysAB was adopted by generation of an expression vector encoding the *hysB* gene followed by a Strep tag and the *hysA* gene lacking the first eleven aminoacids residues [12]. To create this vector two segments were amplified by PCR: the *hysB* gene (HysB Exp Vctr Fw – AGGTTGGGAAGCCCTGCAATGCAGTCCCAGGAGGTACCATAT GAGTCTCACAAGGCGTGATTTCGTC and HysB Exp Vctr Rev – TTTTCGAACTGCGGGTGGCTCCACATG ATATCCTCCTGAAGCGACTGACGG) and the Strep Tag with the *hysA* gene (HysA Exp Vctr Fw – TGGAGCCACCCGCAGTTCGAAAAA GGGGCCACCGGCAGGACGACCATC and HysA Exp Vctr Rev–GATCGTGATCCCCTGCGCCATCAGATCCTTGGCTCGCGGCCCTCCC CTTTCATGAT); and then added into pMO9075 background via SLIC. The amplifications products were transformed into *E. coli* α -select Silver Efficiency (Bioline®), and cells were plated on spectinomycin (100 µg/ml)-containing agar plates. The correct plasmid construct was screened by colony PCR and later confirmed by sequencing at the DNA Core Facility at the University of Missouri, USA. The pMOIP03 vector was successfully introduced in IPAR01 by electroporation [9] in MOYLS4 medium containing 400 µg/ml of geneticin and 100 µg/ml of spectinomycin, generating the complemented strain IPMM01. The plasmid was confirmed by PCR amplification of the insert and also by sequencing performed in GATC Biotech, Germany.

4.2.2 Cell growth

All *DvH* strains were cultivated in modified Postgate medium C [13] with an iron concentration of 25 μM , as previously described [14]. This medium is supplemented with lactate or formate-lactate, as electron donors and sulphate as electron acceptor. Medium C is also supplemented with nickel chloride and sodium selenide, both at a concentration of 1 μM , unless otherwise stated.

Small scale growths, where different concentrations of nickel chloride (1 μM , 200 μM and 400 μM) were added, were performed in closed glass flasks containing 20 ml of culture volume, as described in [13]. The *DvH* strains were cultivated in modified Postgate medium C, supplemented with formate an electron donor and a limiting sulphate concentration (3mM). Cells were harvested at late exponential phase and resuspended in 20mM Tris-HCl buffer pH 7.6 and centrifuged at $2,500 \times g$ for 15 min at 4°C , washed with 20 mM Tris-HCl buffer (pH 7.6) and again centrifuged as above. Cells were then disrupted using the BugBuster[®] Protein Extraction Reagent (Novagen[®]) for 20 min and centrifuged at $16,000 \times g$ for 20 min at 4°C .

4.2.3 Purification of IPMM01 NiFeSe Hase

The IPMM01 cells were re-suspended in 20 mM Tris-HCl, pH 7.6, except in a modified purification protocol where a 20 mM Tris-HCl, pH 7.6 + 50 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ buffer was used to oxidize the cell extract. IPMM01 cells were disrupted in a French pressure cell, under a N_2 atmosphere to minimize O_2 exposure. The soluble fraction was obtained by ultracentrifugation ($42,000 \times g$, 90 min). Purification of IPMM01 Hases was carried out both under aerobic and anaerobic conditions. Anaerobic purifications were carried out inside a Coy

anaerobic chamber (95% N₂, 5% H₂) using an AKTA™ Prime plus™ system. The soluble fraction was applied to a Q-Sepharose HP column equilibrated with 20 mM Tris-HCl pH 7.6 buffer. A stepwise NaCl gradient was performed and fractions were separated according to their UV-Visible spectra. The fractions containing Hase, which eluted between 200 and 300 mM NaCl, were pooled. The buffer was exchanged to 20 mM Tris-HCl, pH 8.0, by concentrating the fractions with a 30-kDa cut-off ultracentrifugation unit (Amicon Ultra-15 30K NMWL, Millipore, USA) and then loaded onto a column containing Strep®-Tactin resin (IBA GmbH) equilibrated with 50 mM Tris-HCl pH 8.0, 150 mM NaCl (Buffer W). After five washing steps with Buffer W, the recombinant protein was eluted with eluting buffer (Buffer W + 2.5 mM desthiobiotin). The eluted fractions were analysed by SDS-PAGE, activity-stained Native-Page and Western Blot with Strep-Tactin horse radish peroxidase (HRP) conjugate. Proteins were not frozen but used directly for experiments, after purification, except for the mass spectrometry measurements.

4.2.4 Construction of IPMM02 U489C

The amino acid exchange U489C in the large subunit (HysA) of the DvH NiFeSe Hase was generated by the NZYMutagenesis kit using the mutagenic primers 5' -TGACGGCACAGCCCAGGCACGGGTCTGAAGGCGCG-3' and 5' -CGC GCC TTC GAC CCG TGC CTG GGC TGT GCC GTG CA-3. Plasmid pMOIP03 carrying a *hysA_{Strep}hysB* fragment was used as the template. The amplification products were transformed into *E. coli* α -select Silver Efficiency (Bioline®) and the new plasmid construct named pMOIPM02 was screened by colony PCR and sequenced at GATC Biotech, Germany. The pMOIPM02 vector was introduced

by electroporation into strain IPAR01, generating the complemented strain IPMM02.

4.2.5 Purification of IPMM02 U489C

Strain IPMM02 was cultured in medium C supplemented with selenium (to suppress expression of the other *DvH* Hases and direct expression of the U489C), as described above. The IPMM02 cells were broken in a French pressure cell, under a N₂ atmosphere to minimize O₂ exposure. Purification of the IPMM02 U489C Hase was carried out both under aerobic and anaerobic conditions using the same protocol as described in 4.2.3. The fractions containing IPMM02 U489C Hase eluted between 200 and 300 mM NaCl, and were identified by Western blot. The final eluted fraction was analysed by SDS-PAGE, Native-Page and Western Blotting. Proteins were not frozen but used for the experiments directly after purification, except in the mass spectrometry measurements.

4.2.6 Analytical methods

Protein concentration of purified fractions was determined with the Bicinchoninic Acid assay from Pierce, using bovine serum albumin as standard. Total protein concentration of crude, soluble and membrane cell extracts was determined by the Bradford assay. Metal content (Fe and Ni) was determined by inductively plasma emission analysis at the Atomic Spectroscopy Laboratory of REQUIMTE (see Appendix Table A.3).

4.2.7 Western blot analysis

The crude cell extracts were run in a 12% SDS PAGE gel with 0.1% Triton X-100. Proteins were then transferred to 0.45- μ m polyvinylidene difluoride membranes (Roche) for 1 h at 100 mV and 4°C in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were equilibrated with Tris-buffered saline solution with Tween 20 (TBST; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), and then treated with antiserum raised against the DvH NiFeSe Hase. The antibody was diluted 1:500 in TBST. Immunodetection was performed by treatment with anti-mouse immunoglobulin G (heavy plus light chain) alkaline phosphatase conjugate (from Sigma) diluted 1:5000, followed by a solution of nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP solution; Roche).

4.2.8 H₂ production by Gas Chromatography

Hase activity was routinely assayed by H₂ production as previously described [15] unless otherwise stated. The H₂ content in the headspace of the serum bottles was determined using a Trace GC 2000 gas chromatograph (Thermo Corporation) equipped with a MolSieve 5A 80/ 100 column (Altech) and a thermal conductivity detector. Nitrogen was used as carrier gas at a flow rate of 10 mL.min⁻¹ and specific activities were determined in a protein concentration range where linearity of activity with protein concentration was established. The activity of the crystallized protein was measured immediately after dissolving crystals harvested from the crystallization drop in 50 μ l of 20mM Tris-HCl pH 7.6 buffer.

4.2.9 Hase uptake activity assays

IPMM01 NiFeSe and reconstituted IPMM02 U489C Hases H_2 uptake activities were measured in a spectrophotometric assay in a Coy anaerobic chamber (95% N_2 , 5% H_2) with methyl viologen (MV) as the electron acceptor, according to [16]. The purified enzyme was activated for approximately 1h at RT in an anaerobic vial containing 1 mL of 50 mM Tris-HCl buffer at pH 7.0 with 1 mM MV and traces of dithionite. The H_2 -oxidation activity was measured at 37°C in a cuvette containing 1 mL of 50 mM Tris-HCl buffer pH 7.0 and 2 mM MV with a trace of dithionite to eliminate residual oxygen. The sample vials and buffers were saturated with pure H_2 , after oxygen removal under vacuum. The reaction was started by the addition of 10 μ L of stock solution of activated IPMM01 NiFeSe Hase (5-10 μ M concentration range) and metal-reconstituted IPMM02 U489C Hase (50-100 μ M concentration range), and the rate of MV reduction was measured at 604 nm, with a UV-VIS spectrophotometer (Shimadzu). For the pH activity profile experiment, a mixed buffer prepared in distilled water (previously boiled and degassed with oxygen-free nitrogen), consisting of MES, HEPES, sodium acetate, Bicine, and CHES (5 mM of each), titrated to different pHs using concentrated HCl or NaOH. Specific activities were determined in a range of concentrations where linearity of activity with protein concentration was established. Each enzyme assay was carried out in triplicate. Activity-stained native-PAGE was performed as previously described [15].

4.2.10 Hydrogen/deuterium exchange activity

The H_2/D_2 exchange activity of *DvH* Hase (in 50 mM Tris-HCl pH 7.5

buffer) was measured by membrane-inlet mass-spectrometry, in which a thermostated 37°C anaerobic vessel with magnetic stirring was connected through a 14 µm Teflon membrane to a mass spectrometer (Pfeiffer Prisma). The output signal of the spectrometer for each mass value is proportional to the partial pressure of the corresponding gas in the reaction vessel [17]. The output signal was first calibrated by saturating the reactor solution with 100% H₂ and then by 20% D₂ in 80% Ar (Air Liquide). The D₂/H⁺ assays were performed as previously described [18].

4.2.11 *In vitro* metal reconstitution of IPMM02 U489C Hase

In the first experiments, *in vitro* Ni reconstitution of the U489C Hase was performed by incubating 100 µg of Ni depleted-protein under anaerobic conditions in 200 µl of 20 mM Tris-HCl buffer at pH 8.0 with 1.5 mM NiCl₂.H₂O, at RT for 1.5h, under a H₂ atmosphere. The protein was subsequently washed several times with 20 mM Tris-HCl pH 7.6 buffer, and concentrated up to 160 µM, using Amicon Ultra centrifugal filters 30K (Millipore) for the crystallization trials and pH activity profiles. A second optimized protocol was developed for the ICP and FTIR experiments. After eluting the IPMM02 U489C Hase, five washing steps were performed with 20 mM Tris-HCl pH 7.6 buffer plus 5 mM NiCl₂.H₂O using Amicon Ultra centrifugal filters 30K, to remove traces of desthiobiotin. The protein was subsequently incubated at RT under H₂ with 20 mM Tris-HCl pH 7.6 and 5 mM NiCl₂.H₂O for almost 1h. After the activation step, the IPMM02 U489C Hase was washed five times with 20 mM Tris-HCl pH 7.0, 1mM EDTA and concentrated with a 30-kDa cut-off ultracentrifugation unit (Amicon Ultra-15 30K NMWL, Millipore, USA).

4.2.12 FTIR spectroscopy and FTIR spectroelectrochemical experiments

Hase samples in Tris-HCl buffer pH 7.6 were first concentrated to 80 μM by ultrafiltration for measurements in the transmission liquid cell, or above 80 μM for measurements in the spectroelectrochemical cell. The IR spectra were recorded at 2 cm^{-1} resolution and averaging 1024 scans using a Nicolet 860 Fourier transform spectrometer, equipped with a mercury cadmium telluride detector and a purge gas system (Whatman) for removal of CO_2 and H_2O . A transmission cell with CaF_2 windows and 82- μm path length was used for standard FTIR measurements. For FTIR spectroelectrochemical experiments the cell with 8- μm path length described in [19] was used. An enzyme solution in 20 mM Tris buffer at pH 8.0, 0.2 M KCl, and a mixture of redox mediators, 0.05 mM each, were added to the cell. The redox mediators used were: indigo tetrasulfonate ($E_0^0 = -76$ mV at pH 8.0, Aldrich), indigo carmine ($E_0^0 = -163$ mV at pH 8.0, Fluka), anthraquinone-1,5-disulfonic acid ($E_0^0 = -234$ mV at pH 8.0, ICN Pharmaceuticals), anthraquinone-2-sulfonate ($E_0^0 = -277$ mV at pH 8.0, Serva), benzyl viologen ($E_0^0 = -358$ mV at all pH, Sigma). A CV-27 BAS potentiostat for electrochemical control of FTIR measurements was used.

4.2.13 Crystallization and X-ray diffraction data collection

Crystals of the aerobically purified IPMM01 NiFeSe Hase (IPMM01-A) and IPMM02 U489C Hase (IPMM02-A) were obtained aerobically at room temperature by the sitting drop vapour diffusion method, using 20% PEG 1500 (w/v) and 0.1 mM Tris-HCl pH 7.6 as precipitant. These crystals were cryoprotected using a solution containing 20% glycerol, 20% PEG 1500 (w/v)

and 0.1 mM Tris-HCl pH 7.6, flash-cooled in N₂ (l) and placed in a storage Dewar. For data collection, IPMM01-A crystals were transferred to a dry shipper and sent to the ID14-1 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, while IPMM02-A crystals were sent to the PXIII beamline at the Swiss Light Source (SLS) of the Paul Scherrer Institute in Villigen, Switzerland. Crystals of the anaerobically purified IPMM01 NiFeSe Hase (IPMM01-N) and reconstituted IPMM02 U489C Hase (IPMM02-N) were obtained inside an anaerobic Genbox (Biomerieux), after 1 week. These crystals were cryoprotected using a solution containing 20% glycerol, 20% PEG 1500 (w/v) and 0.1 mM Tris-HCl pH 7.6. All anaerobic crystallization drops were prepared inside a Coy anaerobic chamber (95% N₂, 5% H₂). The crystals were quickly harvested in a plastic glove box flushed with N₂ (g), flash-cooled in N₂ (l) and stored. For data collection, the IPMM01-N crystals were transferred to a dry shipper and sent to the ID14-4 beamline at the ESRF. The IPMM02-N crystals were sent to I02 beamline at the Diamond Light Source (DLS), in Didcot, UK. Additionally, crystals of IPMM01 NiFeSe Hase oxidized with K₃[Fe(CN)₆] (IPMM01-OX, Table A.1 Appendix) were grown aerobically, as previously described [2]. These crystals of chemically oxidized NiFeSe Hase were transferred to a dry shipper and sent to the ID29 beamline at the ESRF for data collection. The wavelength chosen for all datasets measured at ESRF, SLS and DLS was above the absorption edge K of the selenium atom. The choice of wavelength for the IPMM01-N dataset was further determined by the need to take the utmost advantage of the diffraction quality of the crystal while circumventing the geometrical limitations imposed by the detector and the beamline. The diffraction images were indexed and integrated using XDS [20] and conversion of intensities to structure factor amplitudes was carried out

with CTRUNCATE in the CCP4 program suite [21]. The data collection and processing statistics are listed in Table 4.1.

Table 4.1 Data collection and processing statistics

	IPMM01-A	IPMM01-N	IPMM02-A	IPMM02-N
Beamline	ESRF ID14-1	SLS PXIII	ESRF ID14-4	DLS I02
Detector	ADSC Q210	PILATUS 2M-F	ADSC Q315r	PILATUS 6M-F
Wavelength (Å)	0.9334	0.7514	0.9701	0.9795
Data Processing	XDS/CCP4	XDS/CCP4	XDS/CCP4	XDS/CCP4
Space Group	C2	C2	C2	C2
Unit cell parameters (Å, °)				
<i>a</i>	106.22	106.28	106.20	106.32
<i>b</i>	63.46	62.77	62.66	62.85
<i>c</i>	111.08	110.86	109.84	110.82
β	105.15	105.11	104.97	105.18
Resolution (Å) ^a	54.0-1.30 (1.32-1.30)	45.5-0.95 (0.97-0.95)	45.36-1.40 (1.42-1.40)	29.2-1.04 (1.07-1.04)
Nr. observations	627238 (11212)	5738007 (249114)	549100 (22919)	947376 (57471)
Unique reflections	168785 (5667)	440915 (21734)	136125 (5928)	326275 (21974)
Multiplicity	3.7 (2.0)	13.0 (11.5)	4.0 (3.9)	2.9 (2.6)
Completeness (%)	96.1 (65.5)	100 (100)	99.4 (88.4)	96.8 (88.2)
R-merge (%) ^c	6.0 (31.1)	6.3 (143.0)	8.2 (72.3)	4.1 (58.8)
<I/σ (I)>	13.7 (2.2)	22.3 (1.6)	11.6 (2.0)	16.1 (1.6)
R-meas (%) ^d	6.6 (40.8)	6.5 (149.8)	9.4 (83.8)	4.2 (68.4)
Wilson B (Å ²)	8.4	6.9	8.3	6.9
CC 1/2	1.00 (0.86)	1.00 (0.59)	1.00 (0.61)	1.00 (0.63)
Z ^e	1	1	1	1
Estimated V _M ^f	2.05	2.03	2.01	2.03
Estimated Solvent Content (%) ^f	40.1	39.4	38.7	39.5
PDB accession code				

^a Values in parentheses refer to the highest resolution shell. ^b The high resolution limit was restricted due to spatial overlaps caused by the long cell axis in an unfavourable orientation. ^c R-merge = merging R-factor, $(\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|) / (\sum_{hkl} \sum_i I_i(hkl)) \times 100\%$ ^d R-meas = redundancy independent R-factor, $\sum_{hkl} [N/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / (\sum_{hkl} \sum_i I_i(hkl)) \times 100\%$ [22]. For each unique Bragg reflection with indices (hkl), *I_i* is the *i*-th observation of its intensity and *N* its multiplicity.

^e Nr. molecules in the asymmetric unit.

^f According to Matthews coefficient [23].

4.2.14 Structure determination and refinement

The crystal structures were determined by the molecular replacement method with PHASER [24] via the CCP4 Graphics User Interface [25]. The coordinates of the protein chains of the large and small subunits of the previously published crystal structure of DvH NiFeSe Hase (PDB 2wpm) [2] were used as search models, after removal of all non-protein atoms. Following a quick initial refinement with REFMAC [26], COOT [24] was used to perform model corrections and addition of the active site metals and Fe-S clusters. Refinement was continued with PHENIX [27], consisting of three macrocycles with refinement of positional coordinates, individual isotropic atomic displacement parameters for all non-hydrogen atoms and also refinement of the anomalous dispersion parameters for the Se atom, in the case of IPMM01-A structure. Model inspection and editing was done with Coot [28] against σ_A -weighted $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ electron density maps. Water molecules were added with PHENIX and checked with Coot. Hydrogen atoms in calculated positions were added to the structural model and included in the refinement in riding positions. In the final refinement stages, individual anisotropic atomic displacement parameters for all protein non-hydrogen atoms were refined for all structures. MOLPROBITY [29] was used to investigate model geometry in combination with the validation tools provided in COOT. The refinement statistics are presented in Table 4.2. Figures 4.5 to 4.x were generated using the PyMOL Molecular Graphics System, Version 1.4.1 (Schrödinger, LLC).

Table 4.2 Refinement statistics

Dataset	IPMM01-A	IPMM01-N	IPMM02-A	IPMM02-N
Resolution limits (Å) ^a	51.8-1.3 (1.35-1.30)	45.5-0.95 (0.96-0.95)	42.8-1.40 (1.45-1.40)	29.2-1.04 (1.08-1.04)
R_{work} ^b	0.114 (0.212)	0.105(0.249)	0.112 (0.230)	0.110 (0.258)
R_{free} ^c	0.136 (0.237)	0.116 (0.262)	0.144 (0.276)	0.127 (0.272)
ML coordinate error estimate (Å) ^d	0.10	0.06	0.10	0.07
<i>Model composition and completeness</i>				
Regions omitted	12B	1A-2A, 12B-13B	12B-13B	12B-13B
Non-hydrogen protein atoms ^e	6094	6064	6098	6169
Ligand/ion	46	61	45	36
Solvent molecules	856	878	710	987
<i>Mean B values (Å²) ^f</i>				
Protein	14.4	10.4	12.8	11.5
Ligand/ion	11.3	11.7	8.3	8.6
Solvent	28.4	25.2	26.3	27.0
<i>Model r.m.s. deviations from ideality</i>				
Bond lengths (Å)	0.013	0.009	0.011	0.011
Bond angles (°)	1.618	1.440	1.380	1.537
Chiral centres (Å ³)	0.089	0.090	0.082	0.095
Planar groups (Å)	0.008	0.009	0.008	0.010
<i>Model validation ^g</i>				
% Ramachandran outliers	0.13	0.13	0.13	0.12
% Ramachandran favored	97.6	97.7	97.6	97.5
% Rotamer outliers	0.6	0.8	1.2	1.2
C ^α outliers	3	3	0	0
Clash score	1.8	2.1	1.9	2.0

^a Values in parentheses refer to the highest resolution shell; ^b $R_{\text{work}} = (\sum_{\text{hkl}} ||F_{\text{obs}}(\text{hkl})| - |F_{\text{calc}}(\text{hkl})||) / (\sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl})|) \times 100\%$; ^c The Bijvoet pairs were treated as separate reflections due to the refinement of the anomalous dispersion coefficients for the Se atom; ^d R_{free} is calculated as above from a random sample containing 5% of the total number of independent reflections measured.;

^e Maximum-likelihood estimate by PHENIX; ^f Including atoms in the alternate conformations of disordered groups of residues; ^g Calculated from isotropic or equivalent isotropic B-values;

^h Calculated with MolProbity [29].

4.2.15 Electrochemical studies

Electrochemical experiments were run in a three-electrode glass cell with a saturated calomel reference electrode (SCE) separated from the main compartment in a sidearm connected by a Luggin capillary. The cell temperature was controlled by a water jacket. A platinum wire was used as a counter-electrode. This set-up keeps the reference electrode at room temperature, so the potentials can be converted to SHE by using the correction factor $E_{\text{SHE}} = E_{\text{SCE}} + 0.241 \text{ V}$. All the measurements were performed inside a Mbraun glovebox with an oxygen content below 1 ppm, and the gas used (N_2 from Air Liquide) was flushed through an oxygen filter (Varian) before entering the electrochemical cell. The potentiostat/ was an Autolab PGSTAT30 and a μ -Autolab III electrochemical analyzer controlled by GPES software (Eco Chemie). For probing O_2 inhibition during H_2 production by the *DvH* NiFeSe Hase, the enzyme was poised at low potential (-460 mV vs. SHE) and covalently bound to a 4-aminothiophenol-gold rotating electrode under a N_2 atmosphere [30]. Chronoamperometry at fixed potential was performed in phosphate buffer at 30°C and pH 7.6, with a rotation rate of 500 rpm. 15 μM O_2 (after 1000 s) followed by 100 μM CO (after 1685 s) and again 15 μM O_2 (after 1900 s) were injected in the buffer. Experiments with recombinant Hase were performed on highly oriented pyrolytic graphite (HOPG) electrodes modified with diazonium salt by reducing nitro groups to amino groups, as previously described [31]. The redox signal observed in cyclic voltammograms (CV's) (approximately 94 mV vs. SHE) corresponds to the hydroxylamine signal. Temperature during experiments was 40 °C, and a 0.1 M phosphate buffer pH 7 was used. The protein immobilization followed the protocol described in [30].

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation of IPMM01 NiFeSe Hase

In *DvH* the NiFeSe Hase is a membrane-associated lipoprotein, where a lipidic group present at the N-terminus of HysA is responsible for its membrane attachment [12]. An expression vector encoding the *hysB* gene, a Strep tag and the *hysA* gene (pmoIP3) is shown in Figure 4.1. The first eleven aminoacids of the HysA N-terminal, containing the lipobox that directs the attachment of the lipid group, were removed, and an N-terminal Strep-tag was incorporated. The vector was successfully inserted into IPAR01 (lacking the *hysA* and *hysb* genes) by electroporation and the resulting strain was designated IPMM01.

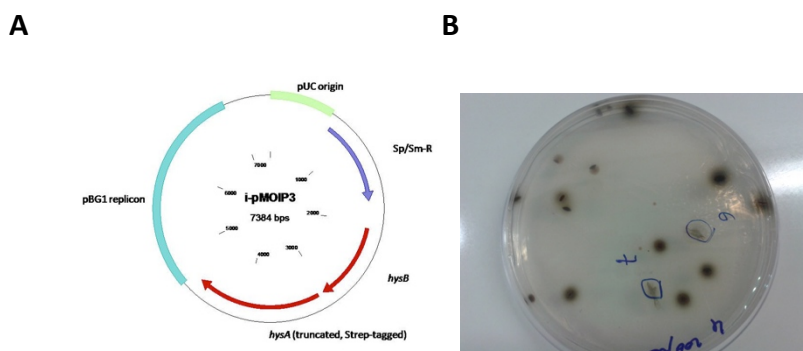


Figure 4.1 The pMOIP03 expression vector (A) was inserted in IPAR01 resulting in the new expression strain, IPMM01, for which colonies are shown (B).

The *wt DvH*, IPAR01 and IPMM01 strains were cultured in small scale under anoxic conditions. Crude cell, membrane and soluble extracts were tested by activity-stained native-PAGE. The IPMM01 strain displayed a band corresponding to the soluble NiFeSe Hase only in the soluble fraction,

confirming expression of the recombinant soluble form of this enzyme. Next, the IPMM01 strain was cultured at a larger scale (300 liters) and an ion-exchange chromatographic step followed by a Strep®-Tactin immunoaffinity column were employed, in the absence of detergents or anaerobic precautions, to isolate the soluble Strep-tagged protein from the crude cell extract. Purification was followed by SDS-PAGE, activity-stained Native-Page and Western Blot analysis, revealing that it was possible to recover active soluble Strep-tagged NiFeSe Hase, as illustrated in Figures 4.2 and 4.3. The IPMM01 recombinant NiFeSe Hase had an average H_2 production activity of 5750 U.mg^{-1} . This compares very well with the reported H_2 production activity of 6908 U.mg^{-1} for the membrane form of *DvH* NiFeSe Hase in a Tris-HCl buffer in phospholipids [15].

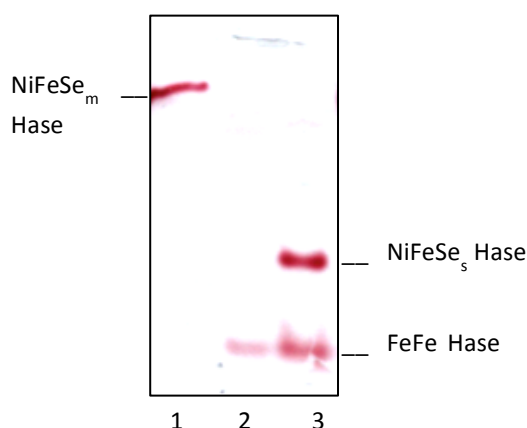


Figure 4.2. Activity-stained native-PAGE of cell extracts (50 μg). Cells grown in medium C enriched with 1 μM Ni and 1 μM Se. **1.** *DvH* wt crude extract; **2.** IPAR01 crude extract; **3.** IPMM01 crude extract.

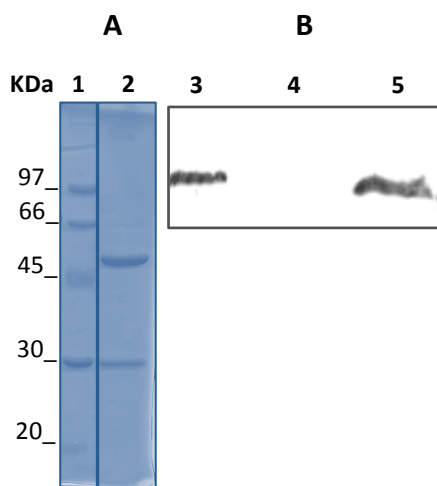


Figure 4.3 **A.** SDS-PAGE stained with Coomassie blue: **1.** LMW Marker; **2.** purified IPMM01 NiFeSe Hase. **B.** Western Blot of crude extracts (10 µg of total protein): **3.** *DvH*; **4.** IPAR01; **5.** IPMM01.

Recombinant NiFeSe Hase was obtained as a homogeneous protein of MW *ca.* 88 kDa, containing the two typical subunits, very similar to the native enzyme, as shown by SDS-Page in Figure 4.3A. Moreover, Western Blot analysis (Figure 4.3B) revealed that the expression levels of the recombinant enzyme are in the same range of the native *DvH* NiFeSe Hase.

The availability of a homologous system to produce recombinant NiFeSe Hase provides a faster source of enzyme, as well as the opportunity to create mutant forms with desired characteristics. A well-established expression system allows the possibility of engineering a soluble Hase resistant to O₂ inactivation, with improved thermostability, increased O₂ tolerance and perhaps with higher H₂ production activity.

4.3.2 Isolation of IPMM02 U489C

The IPMM02 strain, expressing the U489C Hase, was cultured in small scale. IPAR01 and IPMM01 were used as control strains. The IPMM02 strain displayed no Hase in activity-stained Native-Page (Figure 4.4B). The cell extracts were also analysed by Western blotting, as shown in Figure 4.4C. For this, we used the same polyclonal antibodies against the membrane NiFeSe Hase, purified from *DvH*. These antibodies proved to be selective, and confirmed the presence of the mutated protein. A small scale purification using a single chromatographic step (Strep[®]-Tactin immunoaffinity column) was employed, in the absence anaerobic precautions, which yielded pure the IPMM02 U489C, as presented in Figure 4.4A.

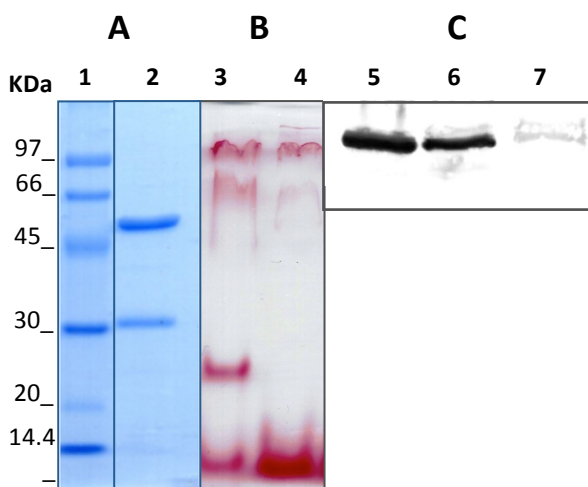


Figure 4.4. **A.** 12.5% SDS-Page stained with Coomassie blue: **1.** LMW Marker; **2.** IPMM02 U489C Hase. **B.** Activity-stained native PAGE of crude extracts (50 μ g): **3.** IPMM01; **4.** IPMM02. **C.** Western blot of cell crude extracts (10 μ g of total protein): **5.** IPMM01; **6.** IPMM02; **7.** IPAR01.

The H₂ production and uptake activities of cell extracts (growth on lactate/sulphate) were also assessed, as listed in Table 4.3. The *in vivo* H₂ production was also quantified and a sharp decrease in this activity by IPMM02 strain was detected (only 21% of the IPMM01 strain activity).

Table 4.3 H₂ production and uptake activities of crude cell extracts measured at pH 7.0 (in units per milligram) and *in vivo* H₂ production by cells grown on formate with limiting sulphate (millilitres of H₂ per litter of cell culture) [13].

Strain	Crude extracts		Cells
	H ₂ production (U.mg ⁻¹)	H ₂ uptake (U.mg ⁻¹)	H ₂ production (ml.L ⁻¹)
<i>DvH wt</i>	5.3	23	900
IPMM01	6.8	64	1120
IPMM02	3.2	5.6	236

In order to understand the molecular basis of this impaired activity, IPMM02 was cultured in large scale (300 L) to isolate the protein. To verify whether the impaired activity could be related to aerobic inactivation during purification, we purified the IPMM02 U489C Hase inside an anaerobic chamber and measured both H₂ uptake and proton reduction activities in crude cell extracts and pure protein. As before, no NiFeSe Hase was detected in activity-stained Native-Page gels of the purified fractions and no H₂ production activity could be measured by GC.

4.3.3 The crystal structure of IPMM01-A, the aerobically purified IPMM01 NiFeSe Hase

The strep-tagged IPMM01 NiFeSe Hase, the soluble recombinant form of the wild type protein, was first isolated and crystallized in aerobic conditions in order to obtain an oxidized form of the protein, with an H_2 production activity of almost 6000 U.mg^{-1} . The crystal form IPMM01-A was obtained with one Hase heterodimer per asymmetric unit, and the structure was solved by molecular replacement using the previously published structure of the oxidized, “as-isolated” soluble NiFeSe Hase from *DvH* (PDB code 2wpm). The structure was refined to $1.3 \text{ \AA}$ resolution, and displayed no major differences in comparison with the native soluble form of the *DvH* enzyme. As previously described [2], the active site is present in three different structural forms, corresponding to the three different conformers observed for the side chain of the Sec 489 residue. In the first two (Sec conformers I and II) an extrinsic sulphur is bound to the Ni atom, as illustrated in Figure 4.5. These two structural forms of the active site represent new motifs for metal binding in Hases [4, 5]. Conformer III of Sec 489 corresponds to an active site structure similar to that found in NiFe Hases and also in the reduced structure of the *Db* NiFeSe Hase [3]. This interpretation is supported by the anomalous difference map calculated from data collected at the high-energy side of the Se absorption edge and phases from the final refined model, showing the three different positions for the selenium atom (Figure 4.6B). In the IPMM01-A structure, conformer I is predominant (53%), followed by conformer III (28%), and conformer II (19%). Also, C75 of the large subunit was found doubly oxidized to $C-SO_2$ (sulphinate). However, and in contrast with the previously reported structures [2, 5], the double oxidation of

C75 was not complete, as judged from the refined occupation factors of the two bound oxygen atoms; the results suggest that C75 is present 64% as sulphinate and 36% as sulphenate. This lower oxidation level of C75 in comparison with the previous oxidized *DvH* NiFeSe structures [2, 5] should be due to the faster purification protocol used for the isolation of the recombinant enzyme. These results also suggest that the oxidation of C75 to sulphinate is a two-step process, with sulphenate as an intermediate. It is known that the thiol side chain of cysteines can be modified in biological systems by reactive oxygen species (ROS) [32] and that the initial oxidation product of Cys is the sulphenate (sulphenic acid, C-SO) that may be stabilized by the protein microenvironment or serve as an intermediate to other reversible or largely irreversible species [7, 32], such as the sulphinate observed in the oxidized crystal structures of *DvH* NiFeSe Hase [2, 5].

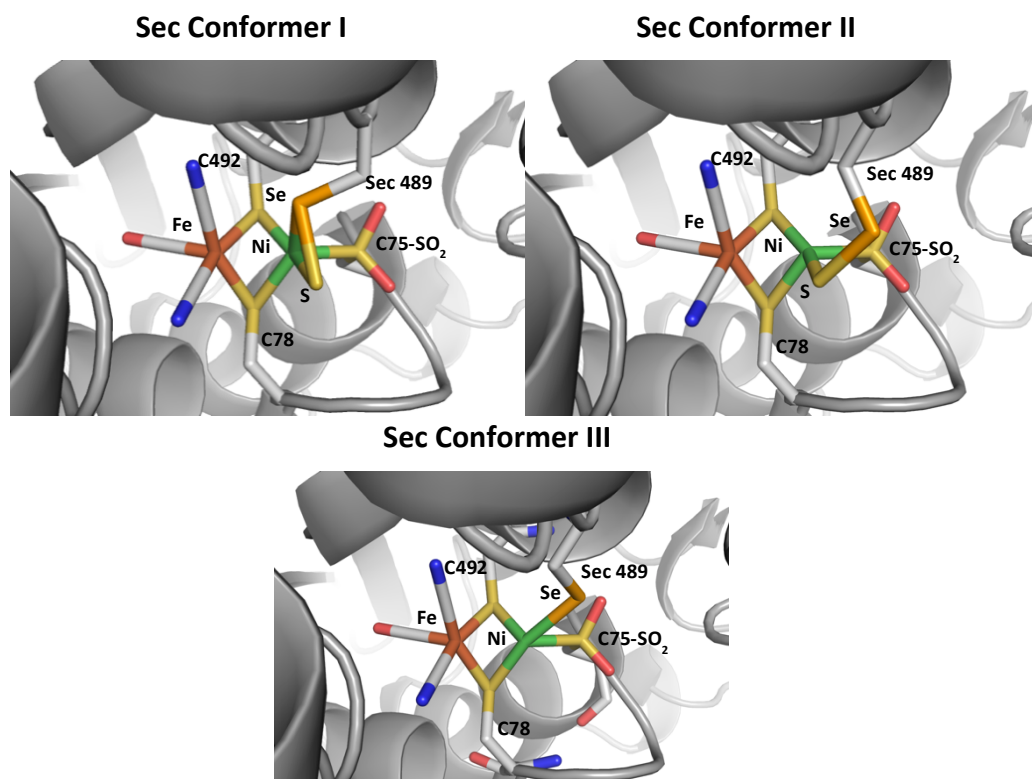


Figure 4.5 Overview of the IPMM01-A active site structure for the three different side chain conformations of Sec 489, denoted by the roman numerals. The NiFe centre and the protein residues are drawn as sticks, with atom colours grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel and gold for selenium.

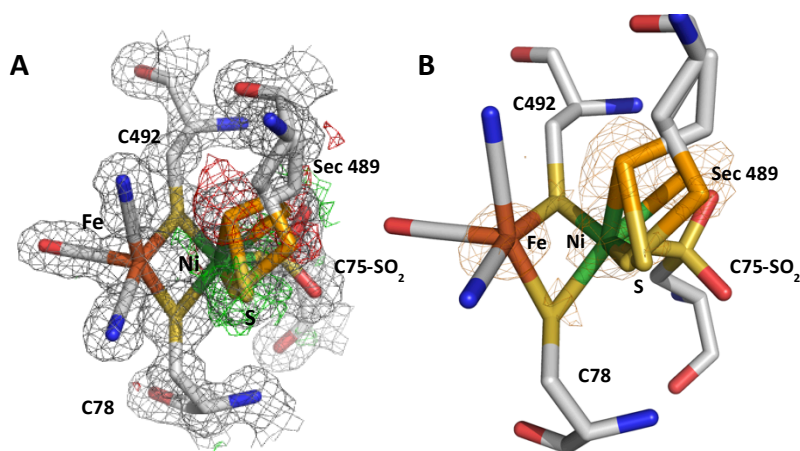


Figure 4.6 Overview of the IPMM01-A active site structure for the three different side chain conformations of Sec 489 **A.** The final $2|F_o|-|F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green mesh) and -3 (red mesh) map r.m.s. levels. **B.** The final anomalous difference map in orange at the 4.5 map r.m.s. level. The NiFe centre and the protein residues are drawn in stick representation, with atom colours grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel and gold for selenium.

Proximal [4Fe-4S] cluster

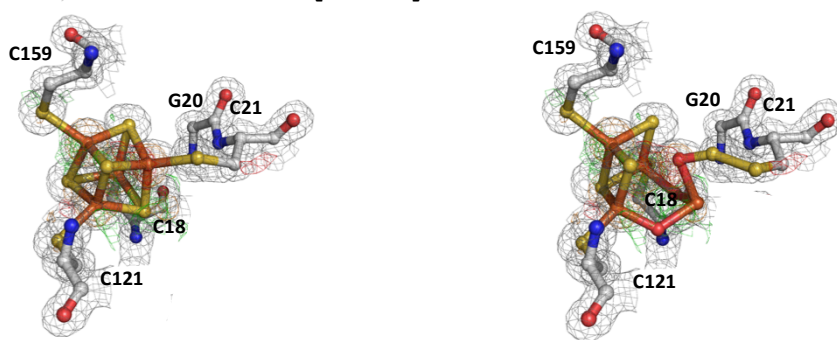


Figure 4.7 Views of the proximal [4Fe-4S] cluster in the two different redox states present in the *DvH* NiFeSe Hase IPMM01-A structure. **Left:** the canonical [4Fe-4S] cubane form; **Right:** the superoxidized [4Fe-4S-3O] form. The [Fe-S] clusters and the protein residues are drawn in ball-and-stick representation. Atom colors are grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur and orange for iron. The final $2|F_o|-|F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green) and -3 (red) map r.m.s. levels.

The proximal [Fe-S] cluster was also found in a partially oxidized form, identified as [4Fe-4S-3O] (Figure 4.7) [5]. Nevertheless, refinement of the super-oxidized component was not possible and instead its concentration was estimated as 22%, based on the refined occupancy factor of the depleted Fe atom in the canonical [4Fe-4S] form. This result confirms the lower oxidative damage of the enzyme, seen both at the active site and the proximal [Fe-S] cluster, due to the faster aerobic purification protocol adopted. Nevertheless, these structural changes still have a critical impact on the Hase catalytic activity. The specific H₂ production measured from the enzyme after crystallization decayed to 1007 U.mg⁻¹, which is only 18% of the initial activity, suggesting that most of the oxidative damage is occurring during the long (5 to 7 days) crystallization process at room temperature. Correlation of these results with the crystallographic data is fundamental to understand the impact of O₂ damage to the redox centres in the enzyme activity, keeping in mind that the structural changes detected in the 3D structures are likely artefacts from the aerobic exposure during purification or crystallization protocols.

4.3.4 The high-resolution structure of IPMM01-N NiFeSe Hase

X-ray crystallographic 3D structures from both native [2, 5] and recombinant *DvH* NiFeSe Hase (4.3.3) were obtained from preparations that contained inactive oxidized states, revealing heterogeneous forms of the active site and proximal cluster. The 0.95 Å resolution structure of the anaerobically purified and crystallized recombinant *DvH* NiFeSe Hase IPMM01-N reported here provides an image of the *DvH* NiFeSe Hase structure in the absence of any irreversible oxygen damage. The enzyme was purified and crystallized under an Argon atmosphere with 5% H₂ and 95% N₂ and crystallized in the monoclinic space group *C2*, with a single heterodimer in the asymmetric unit. Although the protein concentration step was performed aerobically at 4°C, this was a fast procedure and there is no evidence of oxidative damage in the structure. The active site is mostly in the reduced state, which corresponds to conformer III of the previous *DvH* 3D structures [2, 5], and the proximal [4Fe-4S] cluster is in the canonical form. The specific H₂ production activity measured from the enzyme after crystallization was 4300 U.mg⁻¹ (78 % of the initial activity). The first X-ray crystallographic structure of a fully reduced NiFeSe active site was reported for the *Db* NiFeSe Hase purified aerobically, but reduced for over 2 h in a high-pressure cell under 10 bars of pure H₂ [1], and this also showed no evidence of oxidative damage. The absence of unready Ni-A inactive states in NiFeSe Hases could be either related to the O₂ reactivity of the Se atom in the Sec ligand to the Ni, which might undergo a reversible oxidation to seleninate (O=Se=O⁻) [8], or to the different side-chain conformations that can be adopted by the Sec residue in the presence of the extrinsic S atom, blocking access to the NiFe active site by small molecular species such as O₂ [2]. As in the previous *DvH*

NiFeSe Hase structures [2, 5], a possible binding site for the extrinsic sulphur atom in the active enzyme (as an H_2S molecule) is found *ca.* 7 Å away from the active site (Figure 4.8).

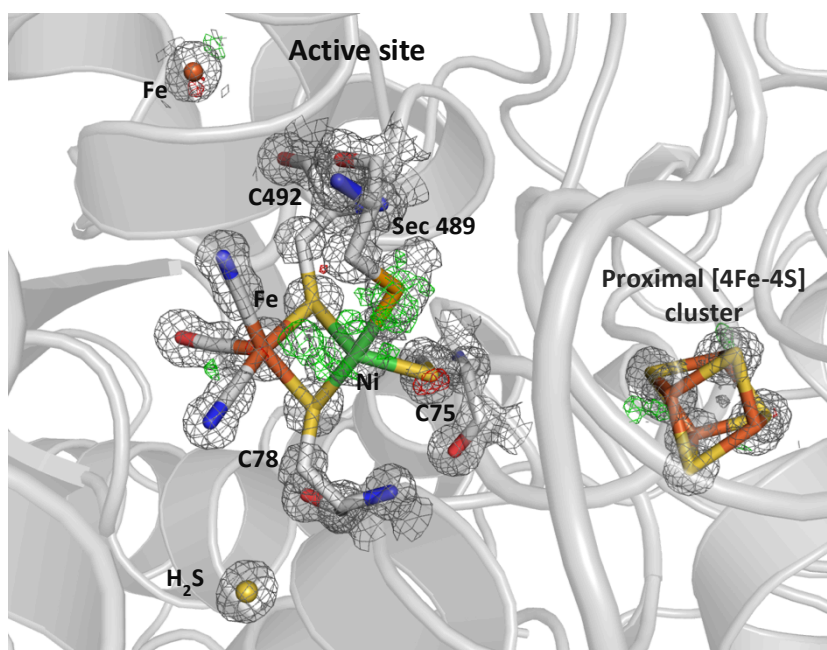


Figure 4.8 View of the IPMM01-N structure showing the NiFeSe active site and the proximal [4Fe-4S] cluster. The protein residues are drawn as sticks, and the H_2S molecule (yellow) is drawn as sphere. Atom colours are grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel and gold for selenium. The final $2|F_o| - |F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o| - |F_c|$ map is represented at the 3 (green mesh) and -3 (red mesh) map r.m.s. levels.

An unexpected result is the presence of two positions for the Ni shifted by approximately 1 Å from each other (Figure 4.9). This observation was only possible due to the near atomic (0.95 Å) resolution of the IPMM01-N dataset. Position I is the most occupied, with 82% Ni bound to the Sec residue that is present in two different conformations: 69% as conformer III, similar to the reduced state in the *DvH* and *Db* NiFeSe Hase structures, and 7% as conformer II (Se-III) described in Figure 4.10, suggesting that the enzyme is not in a fully reduced state. Interestingly, in the previously determined structure of the re-oxidized native enzyme (ReOx24, see chapter 3) Sec489 was also found in conformers III and II, reinforcing the hypothesis that reduction and re-oxidation of the active site follow structurally different pathways. In the less occupied position II the refined occupancies of Ni and Se are 7% and 10% (Se-II), respectively as shown in Figure 4.9.

These active site changes were initially inferred from $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ maps, and later confirmed from anomalous difference maps (Figure 4.9). The occupation factors for all Ni and Se positions were refined independently without imposing any full occupancy constraints. The fact that the occupancy sums for the Ni (89%) and Se (86%) do not add up to 100% is probably an indication of the error associated with the refined values, and may also result from a small effect of their anomalous dispersion parameters, not considered in the refinement calculations. In addition, although clearly seen in the $2|F_o|-|F_c|$, $|F_o|-|F_c|$ and anomalous difference maps, the two Ni and the three Se atomic positions are so close to each other that it was necessary to introduce artificial distance restraints to ensure a well-behaved refinement.

In the previously published *DvH* NiFeSe Hase structures [2, 5] an extrinsic sulphur atom is bound to the Se atom of Sec 489 in conformer II, and

in the new IPMM01-N structure a very weak peak is observed in the final $|F_o| - |F_c|$ map that very likely corresponds to that sulphur atom. This S atom, present bound to Se-III (Ni position I), was not refined because of its very low electron density, expected to be below $0.07 \times 16 \text{ e}^-/\text{\AA}^3 = 1.12 \text{ e}^-/\text{\AA}^3$, i.e., barely above the level of a hydrogen atom, but it is represented in a calculated position in Figure 4.10. Likewise, rather than alternative conformations/positions for the full Sec 489 residue, only the alternative positions, occupancies and atomic displacement parameters of the minor Se sites were refined. Interestingly, two Se sites were also assigned in the *Db* NiFeSe Hase structures. The authors argue that the Ni positions which correspond to the two different Se atom positions could be related to activated/non-activated forms of the enzyme [8]. In addition, a significant conformational change of the Ni surroundings was observed in the non-activated form of the protein. There are structural movements of the Ni atoms toward a peptide amide, similar to movements also observed in the proximal [Fe-S] cluster of the O₂-tolerant NiFe Hases [4, 8].

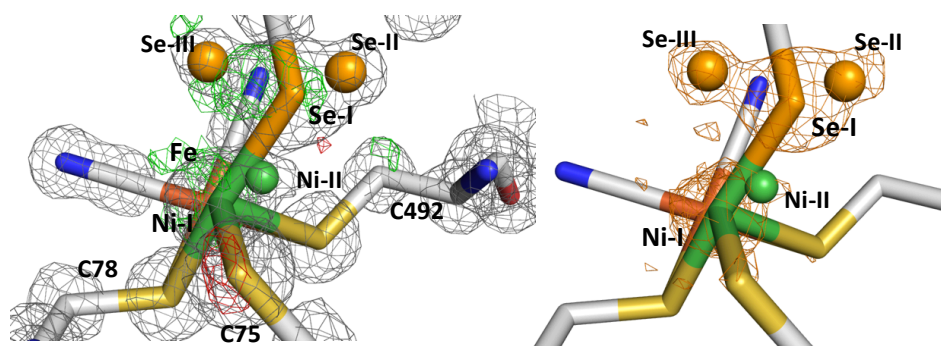


Figure 4.9 The active site in the IPMM01-N *DvH* NiFeSe Hase structure, where the Ni and Se atoms are present in two different positions corresponding to Ni-I and Se-I, Ni-II and Se-II. Additional Ni-II and Se-II atoms are drawn as spheres. The NiFe centres, [Fe-S] clusters and the protein residues are drawn in stick representation with atom colours grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel and gold for selenium. The roman numerals denote the Ni and Se atom in each of the two positions in this crystal structure. The final $2|F_o| - |F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o| - |F_c|$ map is represented at the 3 (green mesh) and -3 (red mesh) map r.m.s. levels. The final anomalous difference map is shown in orange at the 3.5 map r.m.s. level.

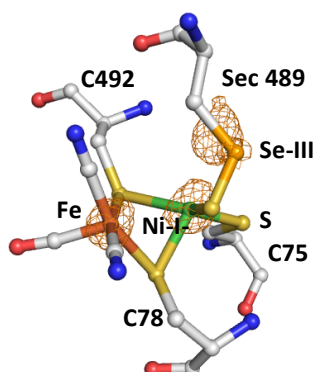


Figure 4.10 View of the active site in the IPMM01-N *DvH* NiFeSe Hase structure showing the minor conformer where Se-III is bound to S (Ni-I and Se-III), which corresponds to the *DvH* NiFeSe conformer II. The NiFe centre and the protein residues are drawn in stick-and-ball representation, with atom colours grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron, green for nickel, gold for selenium.

4.3.5 The impact of Se substitution in the structure of the U489C Hase

In the first crystal structure of the IPMM02 U489C Hase (IPMM02-A) most of the protein molecules lacked Ni at the active site (Ni occupancy refined to 17%). Instead, a putative $\text{H}_2\text{S}/\text{HS}^-$ (83% occupancy) species was found in place of the missing Ni atoms (Figure 4.11). The identity of this new ligand was validated by its equivalent isotropic thermal motion parameter ($13.1 \text{ \AA}^2$), very similar to those of C489 S^γ ($13.2 \text{ \AA}^2$) and Fe ($9.4 \text{ \AA}^2$). The density was interpreted as $\text{H}_2\text{S}/\text{HS}^-$ due to the lack of a Ni peak in the anomalous difference map and a HS^- ligand would contribute to stabilize the positive charge of the iron atom and also complete the octahedral coordination of the iron. The $\text{H}_2\text{S}/\text{HS}^-$ ligand may also be covalently bound to C489 S^γ ($2.1 \text{ \AA}$) since there is no evidence of an alternate conformation for this residue when the Ni atom is absent from the active site. This residue, which is the Sec residue (Sec489) in the wt protein, was found partly oxidized to sulphenate (C-SO) and sulphinate (C-SO₂) as assessed from the refined occupation factors of the bound oxygen atoms (83% and 66%). As in the case of C75 in the IPMM01-A structure, these results suggest that the oxidation of C489 to sulphinate is a slow and sequential process with sulphenate as an intermediate. Oxidation of the Sec residue (corresponding to C489) to seleninate (SeO₂) was reported for the *Db* NiFeSe enzyme [8], but was never observed for any *DvH* NiFeSe structure. Volbeda and co-authors proposed that this oxidation occurred after exposing anaerobic crystals to O₂ [8]. The seleninate may not have been present in *DvH* was not because the S bound to Se atom prevents the reaction with O₂. *DvH* and *Db* are both SRB and produce sulphide in their respiratory metabolism, therefore the presence of an

H₂S/HS⁻ ligand may be explained by the fact that there is a high concentration of H₂S in the cell extract that may react with the active site during the initial stages of purification giving rise to Se sulphuration. Nevertheless, it would seem that the reactivity of the two NiFeSe active sites is different, as judged by the absence of a S ligand in the active site of the oxidized *Db* structures [8].

Another striking difference of the IPMM02 U489C Hase structure relative to that of the *wt* NiFeSe Hase is the presence of three different conformations for C75, instead of the C75-SO₂. The first conformation (conformer A) corresponds to an active site with the Ni atom coordinated by the C75 thiolate, although with a longer bond distance (2.5 Å) than usual. Conformers B and C are observed when the Ni atom is not present, and a disulphide bond is possible between C75 and C78, for both C75 conformers (Figure 4.11). The disulphide bond may indicate an intermediate state of the active site before Ni incorporation. An additional spherical blob of electron density was interpreted as arising from an H₂O molecule with partial occupation near C75 S^γ that should be present only when conformer A is not present. Finally, the proximal [4Fe-4S] cluster was also found to be 25% oxidized to [4Fe-4S-O3], as shown in Figure 4.13.

A refined 17% occupancy for the Ni atom and the highly oxidized active site explain the low activity measured for H₂ production (approximately 6 U.mg⁻¹). For the Hase activity assays a high concentration of sodium dithionite was used, which reduces the inactive states of the enzyme that are reversible.

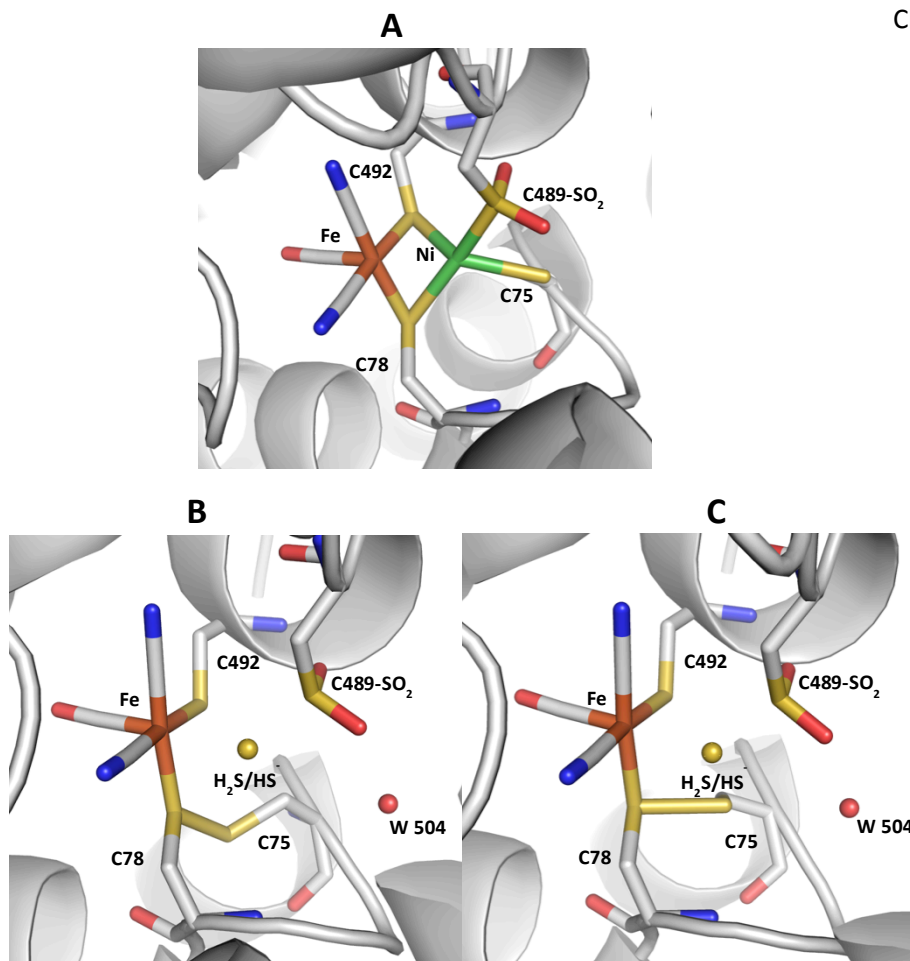


Figure 4.11 Views of the active site of U489C DvH NiFeSe Hase in three different conformations, as observed in the IPMM02-A structure. **(A)** The intact NiFe centre (17% occupancy); **(B)** and **(C)** The active site without Ni (83% occupancy). A water molecule and the H₂S/HS⁻ ligand are drawn as spheres. All the protein residues are drawn as sticks, with atom colours grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron and green for nickel.

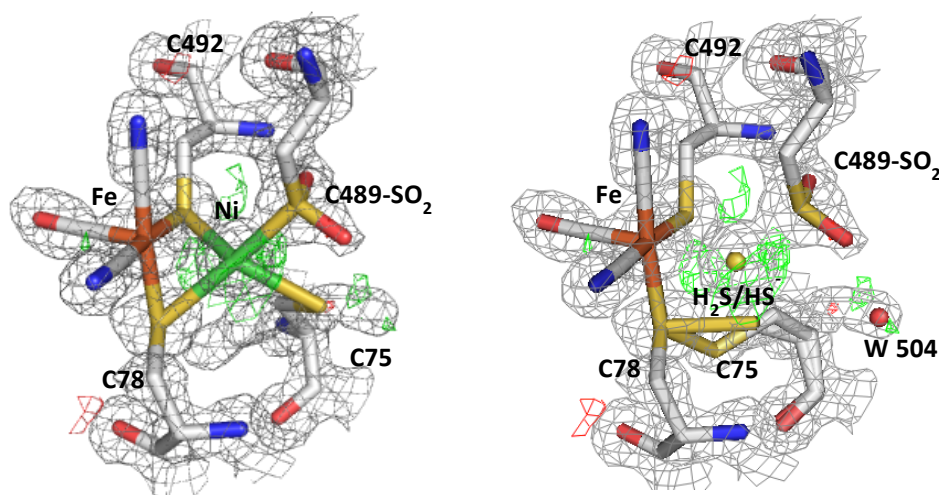


Figure 4.12 Views of the active site in the IPMM02-A structure of U489C NiFeSe Hase. The NiFe center and the protein residues are drawn in ball-and-stick representation, with atom colors grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron and green for nickel. The final $2|F_o| - |F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o| - |F_c|$ map is represented at the 3 (green mesh) and -3 (red mesh) map r.m.s. levels.

Proximal [Fe-S] cluster

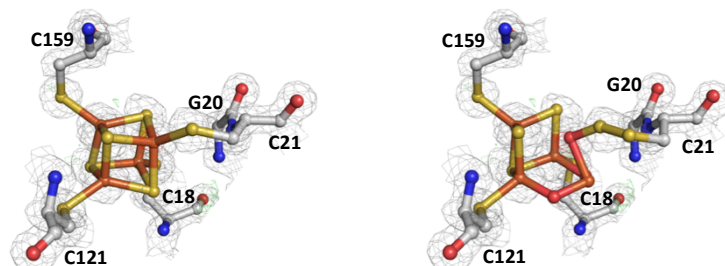


Figure 4.13 Views of the proximal [4Fe-4S] cluster in two different redox states, in the IPMM02-A structure of U489C Hase. The [Fe-S] cluster and the protein residues are drawn in ball-and-stick representation. Atom colors are grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur and orange for iron. The final $2|F_o| - |F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o| - |F_c|$ map is represented at the 3 (green mesh) and -3 (red mesh) map r.m.s. levels.

The biological assembly of the Ni-Fe active site involves a pathway that is not completely established [33–35]. The synthesis and insertion of the $\text{NiFe(CN)}_2\text{CO}$ catalytic centre requires the activity of a complex set of accessory proteins [33]. These maturation proteins seem to be very specific in their action on the target complex. The HypA and HypB proteins are responsible for Ni incorporation/maturation and Ni insertion, respectively [34, 35]. HypA cooperates with HypB in the insertion of nickel into the precursor of the Hase large subunit. The deletion of these genes blocks the protein maturation, which is partially restored by addition of Ni in high concentration [34]. This approach was tested by small-scale growth of IPMM02 cells with increasing Ni concentrations (up to 200 times higher than usual). Still, no effect was detected on restoring the enzyme's activity, either by the activity-stained native PAGE of crude extracts or by measuring the H_2 production activity of IPMM02 cells (Figure A.4 Appendix). The accessory genes present in *DvH* needed for incorporation, insertion and maturation of Ni into the NiFe(Se) Hases, have been identified from sequence comparison with known gene sequences [36]. The presence of genes coding for maturation and regulatory proteins downstream of the structural genes is known for a number of organisms. In *DvH*, both NiFe_1 and NiFeSe enzymes as well as some of their maturation proteins are present in the same gene locus, suggesting a coordinated regulation [12, 37]. In *E. coli* three different NiFe Hases are present and it was demonstrated that they all require distinct sets of maturation genes [33, 38, 39]. The function of these maturation proteins is very specific and one cannot be replaced by another [38]. However, in *DvH* only one set of proteins was identified by sequence analysis. Nevertheless, our results show that the change of a single amino acid residue from Sec to Cys had a drastic effect on

incorporation of Ni, suggesting that at least one of the proteins responsible for these maturation steps needs to recognize Sec to work correctly, which in turn implies that, contrary to present beliefs, different proteins are probably present for this maturation step of *DvH* NiFe and NiFeSe Hases. Thus, the maturation of the *DvH* NiFeSe Hase requires further investigation.

4.3.6 Reconstitution of the Ni-Fe active site in the IPMM02 U489C Hase

The first protocol used to try to reconstitute the Ni-Fe active site of the U489C Hase consisted in incubating the anaerobically purified Ni-depleted U489C enzyme at RT in 20 mM Tris-HCl buffer pH 7.0 with 1.5 mM $\text{NiCl}_2 \cdot \text{H}_2\text{O}$ under H_2 for 1.5h. Subsequently, activity-stained native PAGE was used to assess the activity of the protein, and a band corresponding to U489C could now be detected. Moreover, the H_2 production activity measured for this reconstituted protein increased to $184 \text{ U} \cdot \text{mg}^{-1}$. This activity is in a similar range to that observed for the native *DvH* NiFe₁ Hase [15], indicating that Ni was integrated to considerable extent into the Ni-depleted U489C enzyme active site. Following up on this result, crystallization trials were immediately started with this preparation, to confirm the preliminary results by X-Ray crystallography. The crystal structure of the anaerobically purified and Ni-reconstituted U489C enzyme (IPMM02-N) revealed that Ni was indeed incorporated into the active site in approximately 50% of the molecules. In addition, the anomalous difference map and the high-resolution data allowed the identification of two different positions for the Ni atom in the active site, as illustrated in Figure 4.14 and already detected to a smaller extent in the IPMM01-N structure. As before, the presence of an $\text{H}_2\text{S}/\text{HS}^-$ ligand was also

identified (at approximately 50% occupancy), confirming that the protocol used did not fully restore the Ni-Fe active site. Surprisingly, the anaerobic purification and crystallization did not avoid oxidative damage at the active site, contrary to the results obtained when the same protocol was applied to the IPMM01 NiFeSe Hase. The refined occupation parameters indicated that nearly 60% of C489 was converted to sulphinate and *ca.* 61% of C75 was oxidized to sulphenate. In the IPMM01 NiFeSe structures, anaerobic crystallization had abolished the C75 modification to sulphinate and no seleninate (U489-SeO₂) was detected in any of the *DvH* NiFeSe Hase crystal structures. It is noteworthy that in the “as-isolated” oxidized state of the native *DvH* NiFeSe Hase [2, 5], the active site contained the cysteine residue (C75) as a sulphinate while a seleninate modification of the Sec residue, as reported in the *Db* enzyme, was absent. In contrast, the substitution of the Sec residue for a Cys immediately resulted in this high level of oxidation. This supports the hypothesis that the Sec residue may recover from oxidative damage and that its role may not be strictly catalytic, but also function as protective antioxidant [40]. As already mentioned, the anomalous difference map unequivocally demonstrates two different positions for the Ni atom, 0.9 Å apart from each other (Figure 4.14A and B). The Ni-Fe distance for site A is higher (3.1 Å) than for site B (2.3 Å). There is no evidence in the electron density maps that the Fe atom or the bridging cysteine residues 78 and 492 have significantly different positions for each Ni site. In the reduced structure of *Db* NiFeSe Hase this distance is 2.5 Å, which is 0.4 Å shorter than in the oxidized structure of *Dg* NiFe Hase [1]. As discussed above for the IPMM01-N structure, the nature of these changes in the Ni position remains unclear. However, it can be speculated that the Ni site A corresponds to an inactive form of the enzyme. At lower data resolutions (e.g., below 1.3 Å)

the two Ni sites cannot be resolved in the electron density maps and this heterogeneity probably appears as an “average” Ni position with a higher atomic displacement parameter than that of its more loosely bound Fe neighbour.

No bridging oxide ligand is observed in the anaerobic structure of the IPMM02 U489C Hase, due to the fact that the protein was crystallized in an anaerobic box with a reducing 5% H₂/95% N₂ atmosphere, favouring the presence of the active forms Ni-C and Ni-R. Nevertheless, it is now proposed that besides the hydroxide bridging ligand the Ni-A state contains a sulphenate in one of the bridging cysteines [41]. In 2010, Ogata and co-authors reported the crystal structure of the membrane-associated NiFe Hase from *Av*, where a mono-oxo bridging ligand was assigned between the metal ions of the catalytic centre and one of the bridging cysteines exhibited a modified thiolate [42]. By EPR and FTIR spectroscopies of dissolved crystals it was shown that the molecules in the crystal structure were in the Ni-A state [42].

In the IPMM02-N structure, the proximal [4Fe-4S] cluster was found in the canonical cubane form. Moreover, crystals of anaerobically purified and crystallized U489C that were left to re-oxidize over one week under air (IPMM02-NO), showed only the presence of 25% [4Fe-4S-3O] super-oxidized form (Table A.1 Appendix). There was no evidence for further oxidative damage in these crystals, when compared to the IPMM02-N structure.

Despite the fact that Se and S have similar properties, the selenol group is more acidic than a thiol, which implies that a Sec residue might more easily donate a proton to an acceptor in its vicinity than a Cys residue, favouring, in the case of Hases, the H₂ uptake reaction. Moreover, the Sec to Cys mutation in *DvH* clearly changed the enzyme's inactivation pathway giving rise to an

irreversibly highly oxidized/inactive enzyme with considerably lower catalytic activity, both for H₂ production and H₂ uptake.

In addition to the structural significance of the results presented, it was possible to show that, under *in vitro* conditions, Ni could be substantially incorporated into the active site without the presence of maturation proteins/chaperones, opening up the possibility of *in vitro* maturation of NiFe Hases. The possibility to reconstitute apo-Hases, even in the absence of maturation factors, might result in the development of entirely artificial Hases, with applications as H₂-producing catalysts.

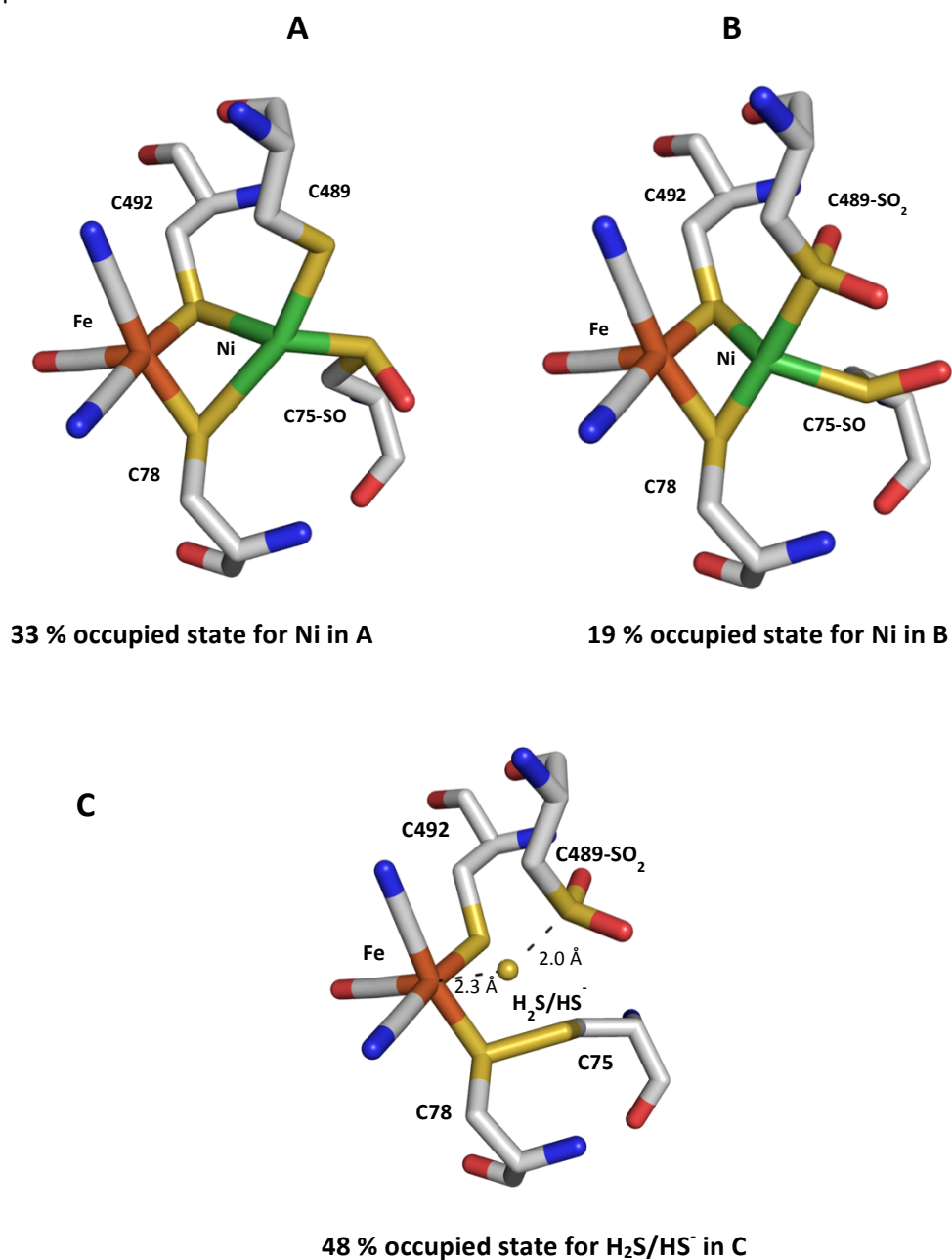


Figure 4.14 Views of U489C NiFeSe Hase active site in three different conformations, as observed in the IPMM02-N structure. **(A)** **(B)** The NiFe centre is drawn in stick representation; **(C)** the H₂S/HS⁻ ligand is drawn as sphere. All the protein residues are represented as sticks, with atom colours grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron and green for nickel.

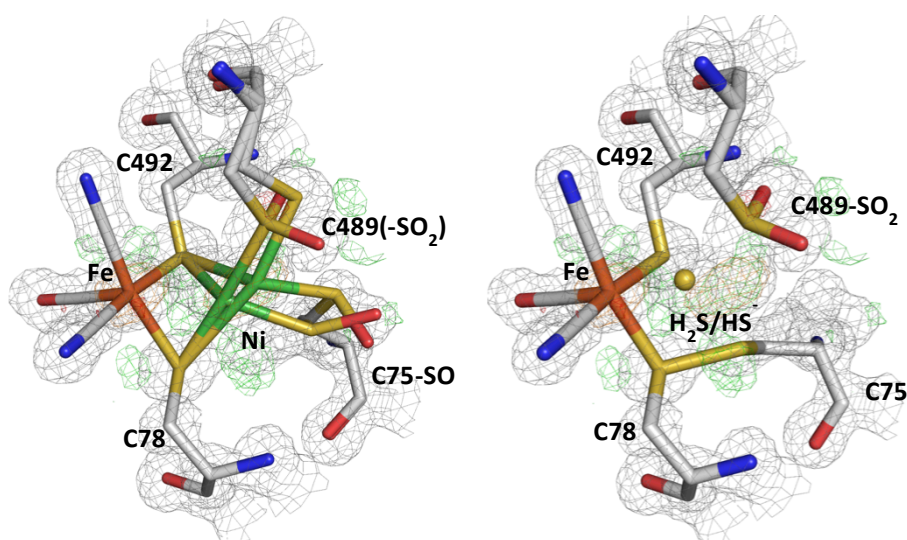


Figure 4.15 Views of the U489C Hase active site in the IPMM02-N structure. The NiFe center and the protein residues are drawn in stick representation. The $\text{H}_2\text{S}/\text{HS}^-$ ligand is drawn as sphere and atom colors are grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur, orange for iron and green for nickel. The final $2|F_o|-|F_c|$ map is drawn as a gray mesh at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is represented at the 3 (green mesh) and -3 (red mesh) map r.m.s. level.

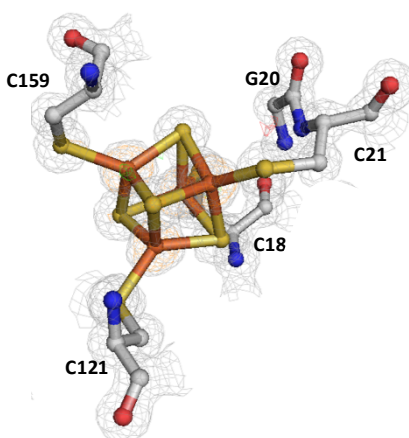


Figure 4.16 View of the proximal [4Fe-4S] cluster in the IPMM02-N structure of U489C Hase. The [Fe-S] cluster and the protein residues are drawn in ball-and-stick representation. Atom colors are grey for carbon, blue for nitrogen, red for oxygen, yellow for sulphur and orange for iron. The final $2|F_o|-|F_c|$ map is drawn in gray mesh at the 1.5 map r.m.s. level and the final $|F_o|-|F_c|$ map is contoured at the 3 (green mesh) and -3 (red mesh) map r.m.s. level.

4.3.7 Enzymatic activities

In solution assays with artificial mediators, NiFeSe Hases usually display higher activities for H₂ production than for H₂ uptake [4, 15, 43, 44]. As the proton is either a substrate or a product in the corresponding reactions, both activities are strongly pH-dependent and different pH profiles are to be expected. The pH dependence of H₂ production and uptake activities has already been reported for the *Db* NiFeSe enzyme [45]. The optimum pH was 7.5 for the H₂ uptake activity with oxidized-benzyl viologen (BV) and pH 4.0 for H₂ production, with reduced-MV. Moreover, it was also observed that the *DvH* NiFeSe Hase had a lower optimum pH than that of NiFe enzymes [4, 18, 44]. This could be due to the pK_a of the Sec selenol group (5.2) that is lower than the corresponding Cys thiol (8.0) [3, 44]. The acidity of the Sec was also suggested to be responsible for the higher H₂ production activities and greater H₂/HD exchange ratio [44]. The pH dependence profiles of the H₂ production activity for the IPMM01 NiFeSe and IPMM02 U489C Hases, are displayed in Figure 4.17. The pH profiles for both enzymes are quite similar, suggesting an optimum pH for H₂ production very close to 6.0, in agreement with the previous results obtained by Gutiérrez-Sanz and co-authors [18] for the membrane-associated *DvH* NiFeSe Hase. It is noteworthy that for the IPMM02 U489C Hase the H₂ uptake activity is higher than its H₂ production activity, in contrast with the Sec-containing analogue (Figure 4.18). Nevertheless, both enzymes display an optimum pH between 10 and 11.

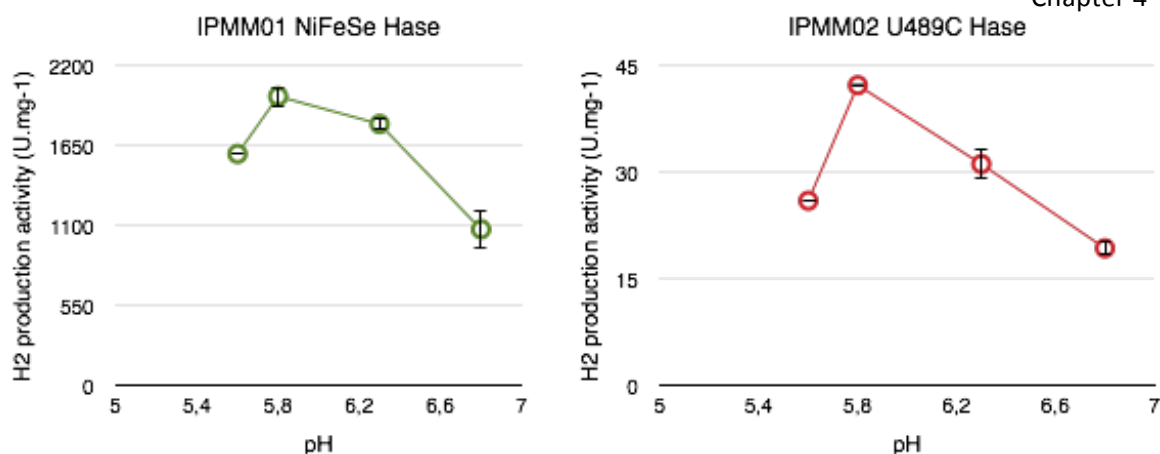


Figure 4.17 pH dependence of the H₂ production activity (U.mg⁻¹) from MV-reduced at 37° C in *DvH* Hases: **A.** IPMM01 NiFeSe Hase; **B.** IPMM02 U489C Hase.

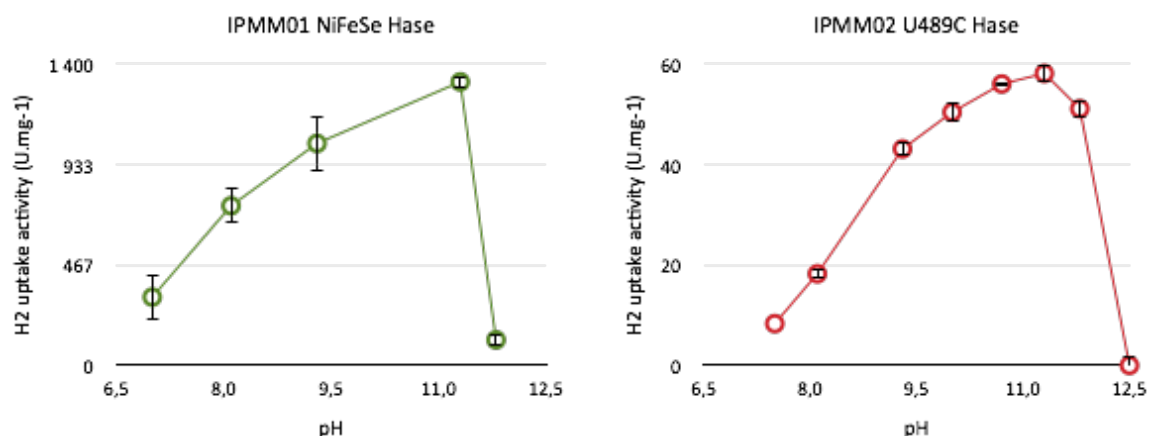


Figure 4.18 pH dependence of H₂ uptake activity (U.mg⁻¹) with MV at 37°C, in *DvH* Hases: **A.** IPMM01 NiFeSe Hase; **B.** IPMM02 U489C Hase.

The very similar shapes of the pH profiles indicate that the different activity behaviour of the NiFe and NiFeSe Hases is determined not only by the properties of the Se and S atoms but also by the overall protein structure, as determined by its amino acid sequence. Indeed, H/D exchange measurements, monitored by mass spectrometry, show that the single exchange/double exchange ratio did not change upon Sec mutation (Table 4.4). This corroborates

the conclusions from a recent study [18], where it is proposed that this performance is not related to the presence of Sec at the active site, but rather to the cage effect of the protein structure surrounding the active site.

Table 4.4 Specific activities of different *DvH* Hases in 50 mM Tris-HCl buffer pH 7.5, 1mM MV and 10 mM dithionite, measured by membrane-inlet mass-spectrometry.

	Turnover rate (s^{-1})		
	<i>wt DvH</i> NiFeSe _m Hase	IPMM01 NiFeSe Hase	IPMM02 U489C Hase
H₂ production	470	1750	45
HD/H₂ (H₂O)	0.4	.	0.5

Based on the turnover rates measured for the *wt* and recombinant *DvH* Hases (Table 4.5) it can be observed that although the highest activity was measured for the membrane form of the *DvH* NiFeSe Hase (approximately $10500 s^{-1}$, in phospholipids), the soluble IPMM01 NiFeSe Hase displays the highest turnover rate in Tris-HCl buffer (Table 4.5), which is stable for long periods (over on month) when the enzyme is kept on ice. This stability was assessed in terms of the catalytic activity and can be further improved if the enzyme is stored on ice under anaerobic conditions. The results support the idea that NiFeSe Hases can sustain extremely high turnover rates and this performance places them as viable candidates for H₂ production applications, as they seem to be biased towards H₂ evolution. The only drawback is that they are still O₂-inhibited, even though NiFeSe enzymes display a higher O₂-tolerance than standard NiFe Hases.

Table 4.5 Maximum specific activities of the different variants of the *DvH* NiFeSe Hase in buffer without detergent or phospholipids.

	Turnover rate (s ⁻¹)			
	<i>wt DvH</i> NiFeSe Hase	IPMM01 NiFeSe Hase	IPMM02 Ni-depleted U489C Hase	IPMM02 Ni-reconstituted U489C Hase
H₂ production	7300	8700	9	270
H₂ uptake	1400 ^[a]	2500	N.D.	90

^[a]From Ref.[15]. Not detected (N.D.)

The unique catalytic properties of NiFeSe Hases confirm their suitability for biocatalysis, as this requires stable, efficient and O₂-resistant catalysts. Indeed, very recently the *Db* NiFeSe Hase was selected as a H₂ evolution catalyst in a solar water-splitting system [46]. Sakai and his collaborators were able to produce H₂ photocatalytically under remarkably high levels of O₂. In addition, a very recent study demonstrated that it is also possible to use O₂-sensitive Hases in a designed biofuel cell where a specific redox hydrogel is capable to protect the enzyme from O₂ damage and high potential inactivation [47]. This enables the construction of new biofuel cells using O₂-sensitive NiFe or NiFeSe Hases.

4.3.8 FTIR spectroscopy

FTIR spectroscopy is a powerful tool to analyse the Hase active site, because of the characteristic CN and CO vibrational stretching modes [48]. The frequencies of these transitions offer an insightful probe of the electron density at the iron centre, and as consequence act as an indicator of the overall density

at the active site. The FTIR spectrum of “as-isolated” oxidized *DvH* NiFeSe Hase revealed the presence of two CO bands (at 1904 and 1939 cm^{-1}) [19]. Bearing in mind the structure of the active site in the as-isolated oxidized form, it was assumed that the active site was present in two different conformations named Ni-IS_I and Ni-IS_{II}, where the first conformation corresponded to the more intense CO band at 1904 cm^{-1} [19]. Subsequently, using other preparations it was observed that the active site could be present in heterogeneous forms, assigned to different conformers of the active site that were related to the redox state of the enzyme [2]. Conformers I and II of the oxidized state were assigned to the Ni-IS_I and Ni-IS_{II}, respectively. The FTIR spectra of the aerobically purified IPMM01 NiFeSe Hase showed that the enzyme is mostly in isoform II of Ni-IS (1941, 2079 and 2094 cm^{-1}), as shown in Figure 4.19. As previously described, isoform II was assigned to the structure of the active site with an exogenous sulphur between Sec489 and the Ni atom (Conformer II) [2]. Upon reduction with H₂, the IPMM01 NiFeSe Hase displayed a mixture of two Ni-R isoforms (CO bands at 1909 and 1935 cm^{-1}). It is not possible to correlate the higher activity of the IPMM01 NiFeSe Hase with a different structure of the active site, because both Ni-R isoforms are present in the active form of the wild-type enzyme.

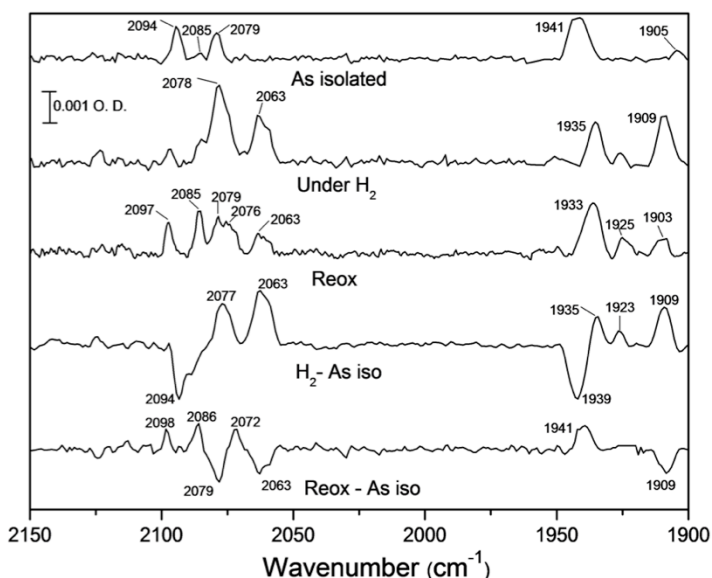


Figure 4.19-Fourier transform IR spectra of the IPMM01 NiFeSe Hase isolated aerobically, reduced under 1 atm H₂ and re-oxidized with air.

The metal reconstitution protocol used for the preparation of U489C Hase for FTIR experiments was slightly different from the one described above, and it led to a slightly higher activity. The samples were purified anaerobically and presented a H₂ production activity of 109 U.mg⁻¹ (pH 7.0), measured by gas chromatography. In the FTIR spectrum of the Ni-reconstituted U489C Hase, shown in Figure 4.21, the peaks were not very intense, although the sample was quite concentrated. These weak peaks in combination with its low specific activity for H₂ production measured by mass spectrometry (30 units.mg⁻¹ at pH 7.5) when compared with the wt NiFeSe Hase, indicated that the IPMM02 U489C Hase did not have a 100% Ni occupancy in the bimetallic active site, as previously described for the IPMM02-N structure. Nevertheless, the FTIR spectra show that the active site bands, in particular the CO bands, are similar

to those of standard O₂ sensitive-NiFe Hases, both in the as “as-isolated” state (resembling NiA/NiB FTIR spectra of NiFe Hases) and after sample reduction under 1 atm of H₂, where the FTIR spectra resembles the Ni-R spectra of NiFe Hases. Re-oxidizing the sample with O₂ resulted in the loss of all FTIR signals, indicating that the U489C Hase is very sensitive to oxidation.

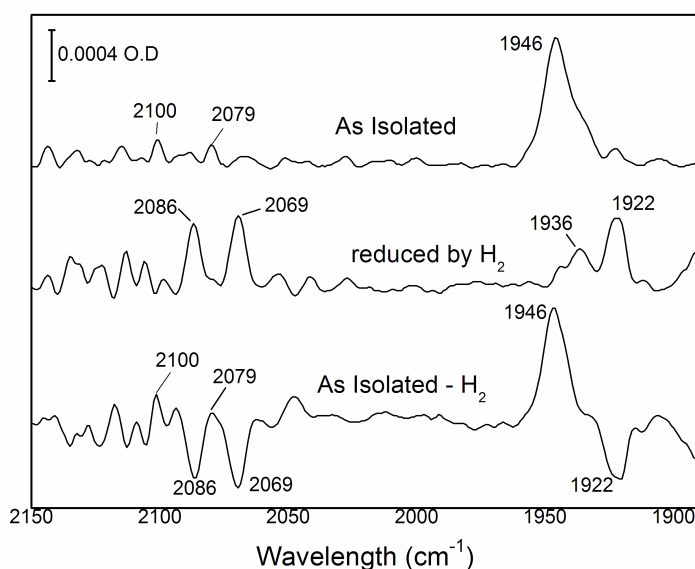
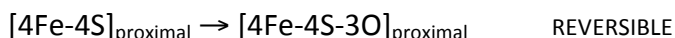


Figure 4.21 Fourier transform IR spectra of IPMM02 U489C Hase as isolated aerobically and reduced under 1 atm H₂.

4.3.9 O₂ inactivation

In order to monitor the oxidative inactivation of the NiFeSe Hase during crystallization, and if the presence of the C75-SO₂ totally abolished enzyme activity, we performed an inactivation profile experiment where we exposed the protein in crystallization drops, without precipitant (as in the sitting drop technique) and measured the H₂ production activity over time. It is important to

mention that in the H_2 production activity assay at 37°C , protein is reduced with sodium dithionite present in the buffer. From the structural results [5] we know that:



There are also structural evidences for the intermediate presence of C75-SOH (sulphenate), but all C75-SOH species should be reduced back to C-SH by sodium dithionite, as it is a reversible species. Thus, the only species present after sample preparation for the H_2 production activity assay should be:



A number of crystallization drops (sitting drops) were prepared aerobically using non-crystallizing conditions (no precipitant in the crystallization drop), at two different temperatures (20° and 4°C), and small aliquots were taken from the drops at regular time intervals. The enzyme inactivation was followed as the decay of catalytic activity over time as represented in Figure 4.22.

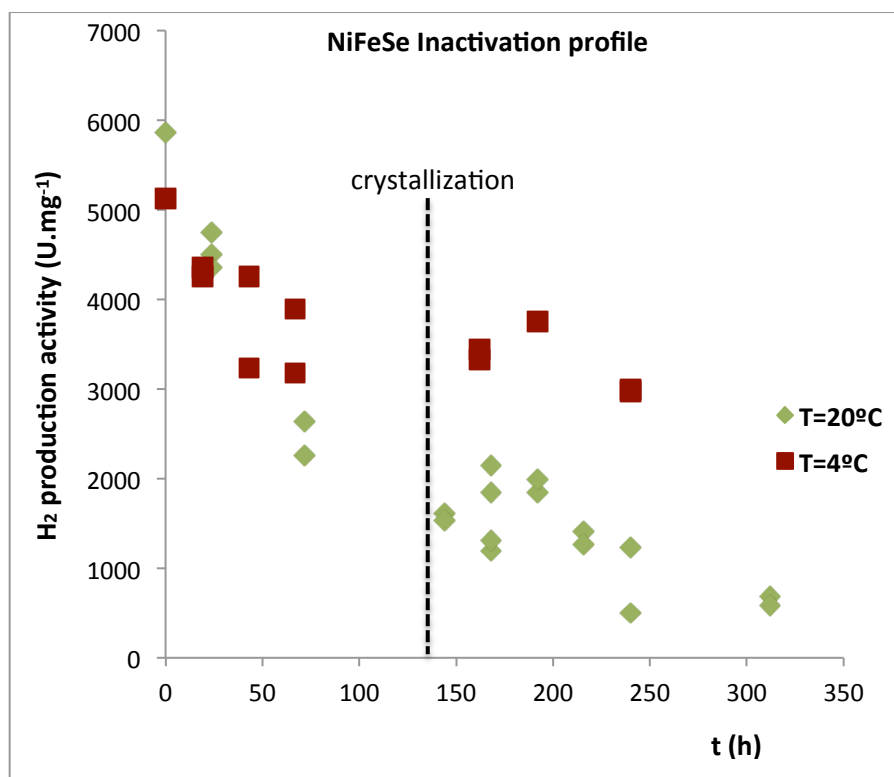


Figure 4.22 H₂ production activity (U.mg⁻¹) decay of IPMM01 NiFeSe Hase upon incubation under aerobic conditions at 20°C and 4°C. Crystallization occurs frequently between 120 and 160h after drop setup and is represent by a dashed line.

It is clear that irreversible enzyme inactivation occurs over time and that it is faster when the Hase is at a higher temperature (20°C), which are the conditions used when for the crystallization. After 312 h at 20°C, which corresponds to almost two weeks, the NiFeSe Hase still displays a H₂ production activity of 635 U.mg⁻¹. This value contrasts with the higher H₂ production activity of 2971 U.mg⁻¹, at 4° C after 240h (an average value of 867 U.mg⁻¹ at 20°C). In another assay, a H₂ production activity of 2260 U.mg⁻¹ was measured after 32 days on ice for the IPMM01 NiFeSe Hase. This result clearly demonstrates that enzyme inactivation over time is strongly temperature-

dependent. Information about the inactivation mechanism might be obtained by analysing the fitting the time-dependence of the catalytic activity decay. However, this analysis only provides information about the irreversible process (C75 oxidation to sulphinate) since the oxidation of the proximal Fe-S cluster to 4F-4S-3O is reversible, as discussed above. The best fit to the 20°C data was obtained from a double exponential decay, suggesting that two independent irreversible Hase isoforms are formed with different rates. Alternatively, FTIR spectroscopy was used to assign the possible inactive species formed during this experiment by continuously measuring FTIR spectra from a sample exposed to O₂, and measuring its activity decay over time in parallel, as illustrated in Figure 4.23.

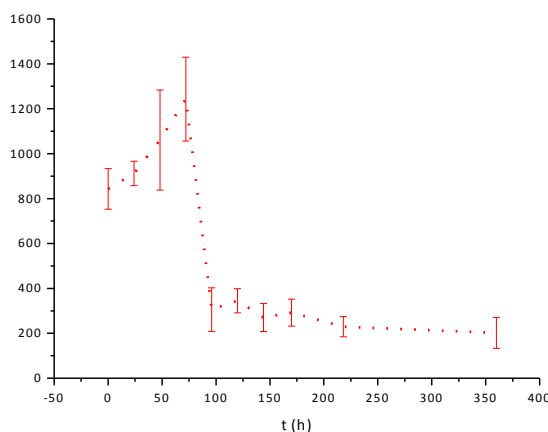


Figure 4.23 Specific activity for H₂ production (U.mg⁻¹) over time (h) measured by membrane-inlet mass-spectrometry, at 37°C in an anaerobic vessel with magnetic stirring, in 50 mM Tris-HCl buffer pH 7.5. The specific activity for H₂ production was in the range of activities measured by mass spectrometry, for a different batch of enzyme: aerobically purified IPMM01 enzyme (624 U.mg⁻¹) and 15 min reduction under 1 atm H₂ (1158 U.mg⁻¹).

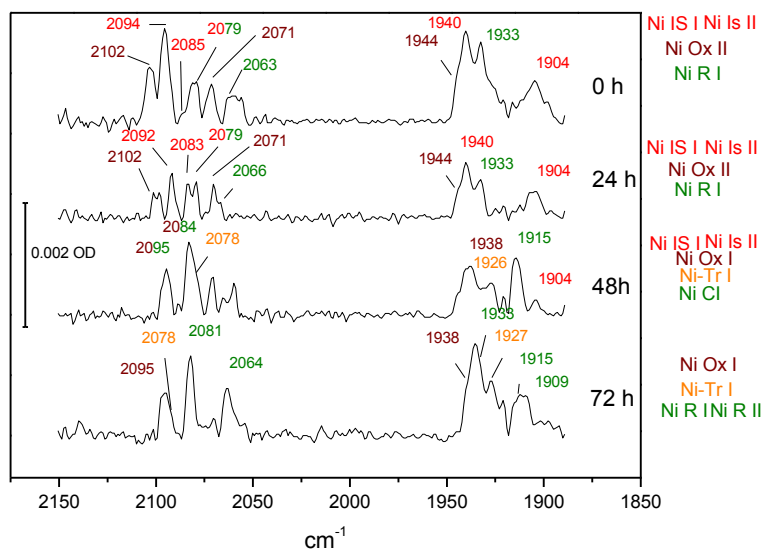


Figure 4.24 Fourier transform IR spectra of IPMM01 NiFeSe Hase under oxidizing conditions over time (exposed to air at RT). Ni IS I and Ni IS II (*as isolated* isophorms I and II), Ni Ox II (reoxidized state of isophorm II), Ni-R I and Ni-R II (reduced states of isophorm I and II, respectively), Ni-Tr I (Ni-transient of isophorm I), Ni-C I (Ni-C of isophorm I). Nomenclature used is consistent with reference [19].

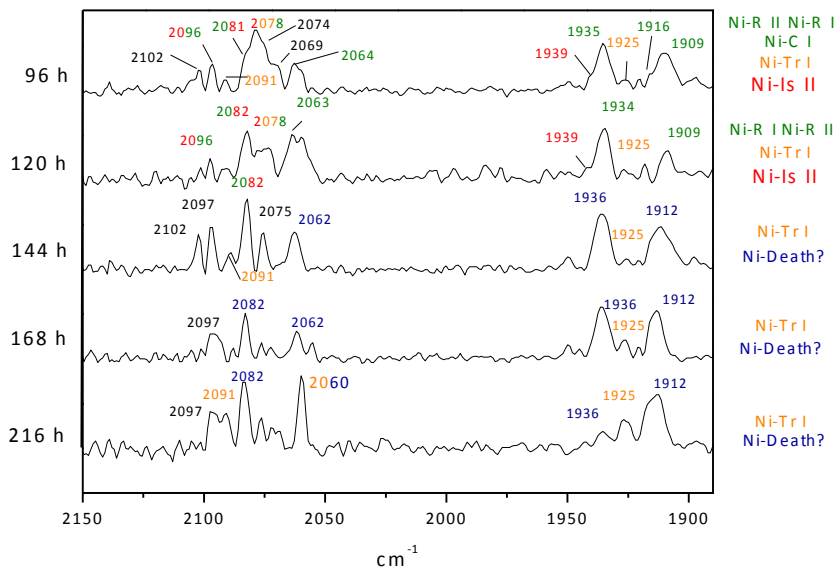


Figure 4.25 Fourier transform IR spectra of IPMM01 NiFeSe Hase under oxidizing conditions over time (exposed to air at RT).

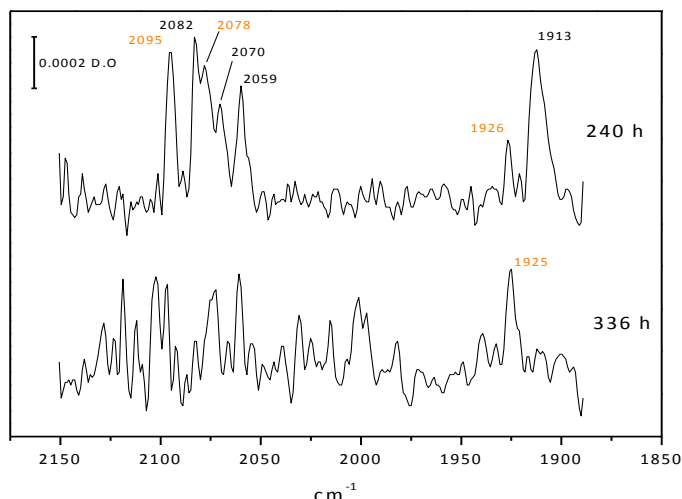


Figure 4.26 Fourier transform IR spectra of IPMM01 NiFeSe Hase under oxidizing conditions over time (exposed to air at RT).

The FTIR spectra are quite complex because there is always a mixture of different species, which probably correlates with the two independent Hase isoforms observed in the oxidized state. At the starting point ($t=0$ s) there is a combination of reduced and oxidized states that is consistent with the purification protocol and the structural data; the sample was purified under anaerobiosis, excluding the concentration step for the FTIR, which was done aerobically (at 4°C). In the first 80 h we can detect a reactivation of the enzyme, reflected in a small increase of the specific activity, which correlates with the presence of reduced states in the FTIR spectra (in particular Ni-C). Afterwards, there is a strong decay of the specific activity, associated with the presence of two FTIR states (CO bands at 1936 and 1912 cm^{-1}) that might correspond to an irreversibly inactivated enzyme (Ni-dead), possibly related to the C75-SO₂ (sulphinat) species present in the 3D structures of *wt* DvH NiFeSe Hase. An unanswered question is whether this sulphinat species totally inactivates the

enzyme, since the inactivation profile with time at 20° still displays a residual activity after 312h.

4.3.10 Electrochemical studies of the *DvH* NiFeSe Hase covalently bound to a 4-ATP gold rotating electrode

The kinetics of O₂ and CO inhibition can be probed using protein film voltammetry (PFV), a technique where the enzyme is adsorbed or covalently bound to an electrode that acts as a sink or a source of electrons. Electron transfer is direct (no mediators are used), and H₂-production activity is measured as a cathodic (reduction) current. This is suitable for detecting rapid changes in turnover rates following addition of inhibitors, as CO and O₂ [49].

Protein film voltammetry studies performed on the *Db* NiFeSe Hase adsorbed on 4-ATP gold rotating electrode reveal that when exposed to small aliquots of O₂-saturated buffer the enzyme inactivation is complete with two kinetically different inactive species [44]. Additionally, in different NiFeSe Hases proton reduction currents were detected in the presence of O₂ [44, 50], a characteristic that was assumed to be exclusive of O₂-tolerant Hases. These results demonstrated that when an electrode-immobilized NiFeSe Hase is exposed to O₂ at negative potentials, a rapid reactivation occurs and the enzyme enters in the catalytic cycle for H₂ production, showing no irreversible damage upon O₂ exposure [4]. This is not the case for standard NiFe Hases.

Figure 4.27 represents an assay where O₂ inhibition during H₂ production by the *DvH* NiFeSe Hase was investigated. It could be observed that after the first injection of 15 µM O₂ the current returned quickly to the initial values (approximately -0.80×10^{-12} µA). The initial large increase in current

intensity is due to the direct reduction of O_2 in the electrode. It is clear that the effect of O_2 on H_2 -production is completely reversible; shortly after its removal the initial catalytic current is reached. The addition of CO, which is a strong inhibitor that binds to the active site, immediately leads to the measurement of the background current in the absence of the hydrogenase activity. The extrinsic CO bound to the active site is also displaced by O_2 , since it can be observed that, after the addition of O_2 following CO, the current increases and returns again to the initial value. Further assays are still need to estimate whether some residual H_2 -production activity remains in presence of O_2 . As for the *Db* NiFeSe Hase, the electrochemical studies illustrate that, under these conditions, the effect of O_2 in H_2 -production is totally reversible.

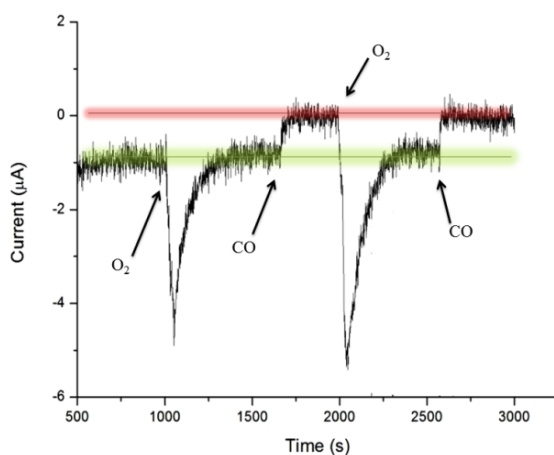


Figure 4.27 Chronoamperometry performed at -460 mV vs. SHE under N_2 atmosphere with a rotation rate of 500 rpm in 0.1M, pH 7.6 phosphate buffer at 30°C with the a gold rotating electrode. The arrows mark the additions of oxygen and carbon monoxide. The straight solid line indicates the stable catalytic current of the enzymatic H_2 -production activity, and the dashed straight line marks the background current with inhibited hydrogenase.

4.3.11 Electrochemical studies of the IPMM01 NiFeSe Hase and IPMM02 U489C on HOPG electrodes

The H_2 production activity of U489C Hase is very low under the experimental conditions (HOPG electrodes and covalent binding to the graphite electrodes modified with aminophenyl groups). The main difference between the two enzymes is that the U489C Hase is quite difficult to activate, and this only happens if sodium dithionite is added to the solution (Figure 4.28B), whereas the IPMM01 NiFeSe Hase (Figure 4.28A) activates fast under H_2 and at negative potentials. On the other hand, we find that both enzymes require a high overpotential for oxidizing H_2 . Under these electrochemical conditions the H_2 production is negligible at the electrode. The shape of the CVs (at a given high rotation rate) indicates a high kinetic barrier for direct electron transfer. This suggests that the orientation of the immobilized enzymes is not the best and further experiments should be done to optimize these experimental results.

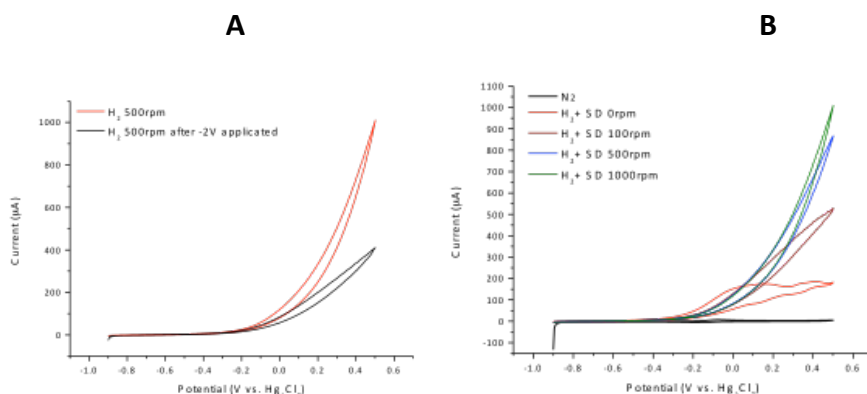


Figure 4.28 Cyclic voltammograms (CV) of HOPG electrodes modified with aminophenyl groups. **(A)** IPMM01 NiFeSe Hase **(B)** IPMM02 U489C Hase. The measurements were made in 0.1 M phosphate buffer at pH 7 and at a 40°C.

4.4 FINAL CONCLUSIONS

In this chapter, we describe a successful *DvH*-based expression system capable of producing a soluble NiFeSe Hase, with a high catalytic activity. The availability of a system to produce recombinant NiFeSe Hases provides a ready supply of the enzyme as well as the opportunity to generate mutant forms with required characteristics. Indeed, we have purified a variant form of the enzyme in which Cys replaces Sec. To elucidate the essential catalytic role of Sec, kinetic and biochemical properties of the variant enzyme (U489C Hase) were compared with those of the recombinant NiFeSe Hase. It was necessary to overcome one problem associated with an ineffective U489C Hase maturation, by incorporating Ni into the active site of the Ni-depleted enzyme via an *in vitro* assay to partially restore the catalytic active site. The mutant and recombinant NiFeSe Hases displayed similar pH dependencies with respect to activity, although the U489C Hase has a turnover number which is more than two orders

of magnitude lower than the Sec-containing enzyme. Both enzymes were inactivated by O₂, judging by the high resolution X-Ray structures; however, the 3D structures of the U489C Hase reveal that the Ni incorporation is not totally efficient and that there is a higher oxidation level of the active site which makes us consider that the Sec has an important role in oxidative protection.

The ability to produce and engineer a soluble NiFeSe Hase, particularly one as catalytic active and as moderately O₂-resistant as DvH NiFeSe Hase, makes this enzyme an exceptional model system for future fundamental and applied studies.

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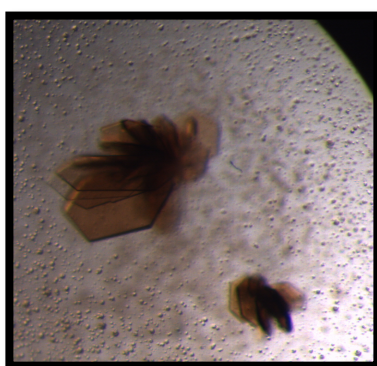
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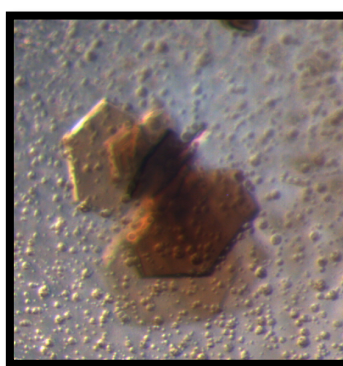
APPENDIX

Table A.1 Preliminary refinements.

Data	IPMM02-NO	IPMM01-Ox1
	DLS, 1.08 Å I02	ESRF, 1.10 Å ID29
Beamline		
Hase Form	IPMM02 U489C	IPMM01 NiFeSe
Purification	Partly anaerobic	Aerobic plus $K_3[Fe(CN)_6]$ addition
Crystallization	Anaerobic + air-exposed for 1 week	Aerobic
% R-work/R-free	10.77 / 12.47	11.43 / 13.43
Coordinate Error	0.07	0.09
Proximal [FeS] cluster	27% oxidized	20% oxidized
Sec489/C489	56% sulfinate	4 conformers
S-link	No	2 positions with 57/22% occupancy
C75	54% sulfenate in 2 conformers; 45% CYS	36% sulfinate
Active site	46% Ni (two positions); 55% HS^-	Two Ni positions with 68/18% occupancy
Solvent molecules	936	744



IPMM01-A



IPMM02-A

Figure A.1 Hase crystals grown aerobically by sitting drop in 20% PEG 1500, 0.1 M Tris–HCl pH 7.5.

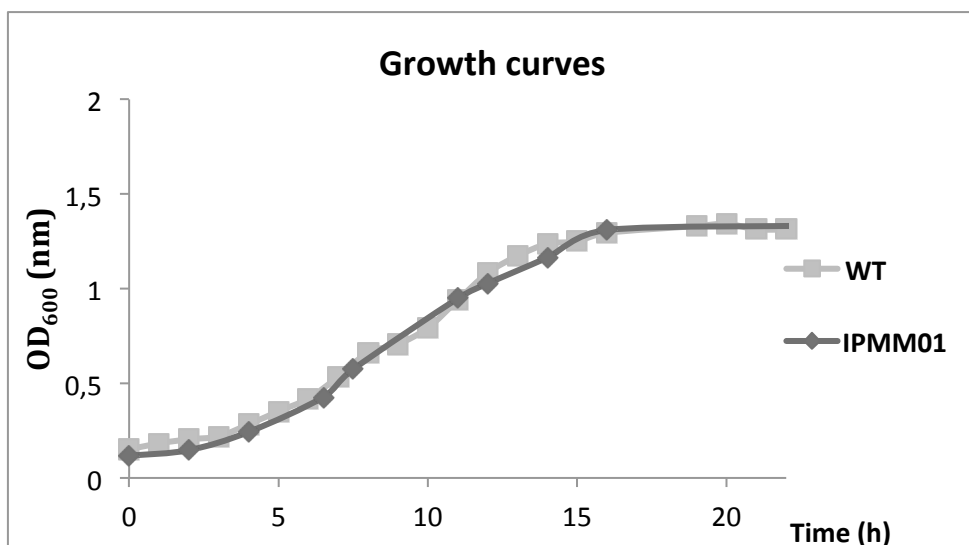


Figure A.2 Growth curves of wt DvH and the IPMM01 mutant strain in medium containing 40 mM lactate and 40 mM sulphate. WT, squares; IPMM01 mutant, rhombus; each data point is the average of two independent growth experiments.

Table A.2 DvH mutant strains carrying engineered variants of NiFeSe Hase, already isolated and sequenced.

STRAIN	Residue	Subunit	Mutations	Role
IPMM02	U489	Large	U489C	Transform in NiFe Hase
IPMA01	G126	Small	G126C	Transform proximal cluster into O ₂ -tolerant-like Hase
IPMM03	E219	Small	E219C	Covalent attachment to gold electrode
IPMM04	E219 V221	Small	E219CV221C	Double covalent attachment to gold electrode
IPMM05	G126G20	Small	G126CG20C	Transform proximal cluster into O ₂ -tolerant-like Hase
IPMM06	V77	Large	V77H	Block entrance to the active site

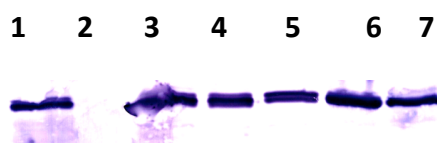


Figure A.3 Western Blots (the amounts used for immunodetection were 20 μ g of total protein): **1.** *DvH* crude extract; **2.** IPAR01 crude extract; **3.** IPMA01 crude extract; **4.** IPMM03 crude extract; **5.** IPMM04 crude extract; **6.** IPMM05 crude extract; **7.** IPMM06 crude extract.

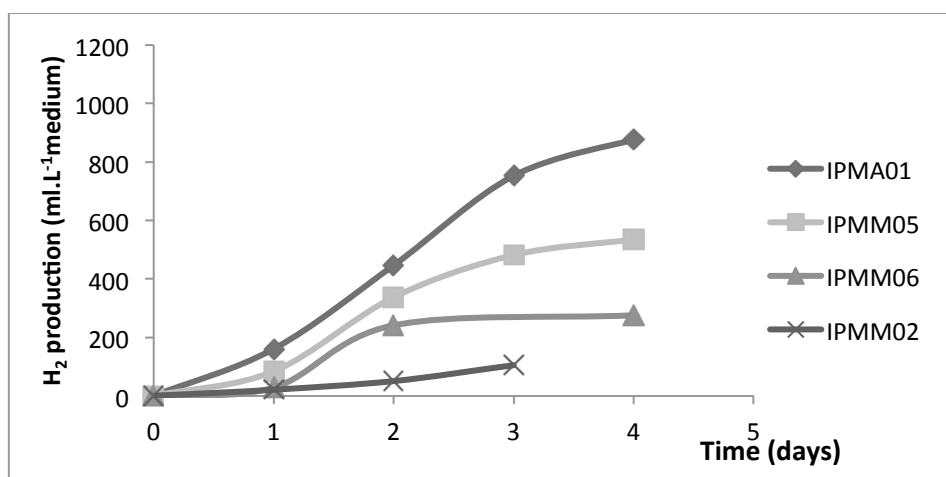


Figure A.4 Hydrogen production by *DvH* mutant strains over four days in the presence of sulphate (3 mM), Fe (25 mM) and Ni and Se (1 μ M), with formate (40mM) as electron donor. Conditions were pH 7.0 and 10% (v/v) cells. Data are the average of triplicate incubations.

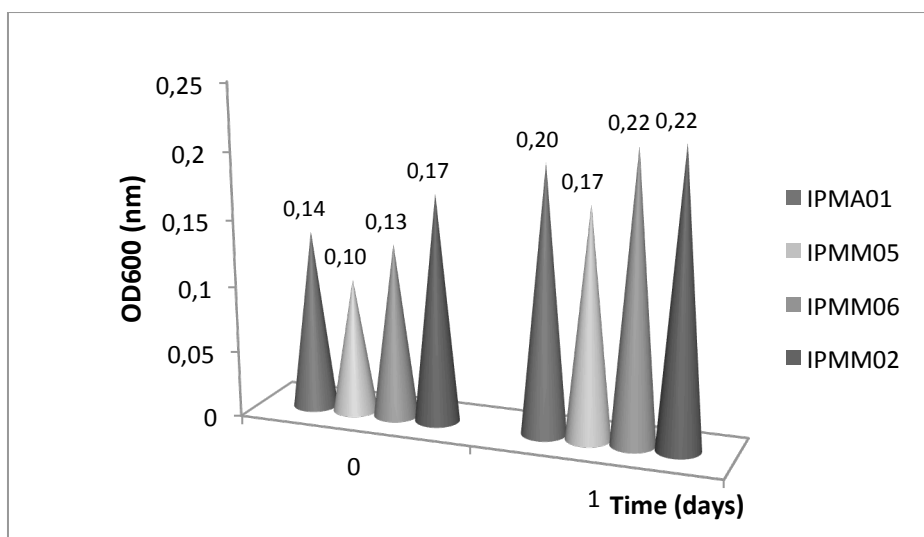


Figure A.5 OD₆₀₀ of DvH strains at time 0 (after inoculating) and time 1 (24 h) growing in the presence of sulphate (3 mM), Fe (25 mM) and with formate (40mM) as electron donor. Conditions were pH 7.0, Ni (1 µM) and Se (1µM) and 10% (v/v) cells. Data are the average of triplicate incubations.

Table A.3 Iron (Fe) and Nickel (Ni) quantification results by ICP. Concentrations are in milimolar (mM).

Sample	Fe (mM)	Ni (mM)	Ratio (Fe:Ni)
DvH membrane NiFeSe Hase	0.015	0.001	15.0
IPMM01 NiFeSe Hase	0.026	0.002	13.0
U489C Hase after in vitro assay with Ni	0.039	0.004	9.8

