DOI: 10.1002/ejic.201001127

Nickel–Iron–Selenium Hydrogenases – An Overview

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Keywords: Hydrogen / Structural Biology / Molecular modeling / Biological activity / Structure–activity relationships

[NiFeSe] hydrogenases are a subgroup of the large family of [NiFe] hydrogenases in which a selenocysteine ligand coordinates the Ni atom at the active site. As observed for other selenoproteins, the [NiFeSe] hydrogenases display much higher catalytic activities than their Cys-containing homologues. Here, we review the biochemical, catalytic, spectroscopic and structural properties of known [NiFeSe] hydrogenases, namely from the Hys (group 1 [NiFeSe] hydrogenase), Fru (F_{420}-reducing [NiFeSe] hydrogenases) and Vhu families (F_{420}-non-reducing [NiFeSe] hydrogenases). A survey of new [NiFeSe] hydrogenases present in the databases showed that all enzymes belong to either group 1 periplasmic uptake hydrogenases (Hys) or to group 3 cytoplasmic hydrogenases (Fru and Vhu) and are present in either sulfate-reducing or methanogenic microorganisms. In both kinds of organisms, the [NiFeSe] hydrogenases are preferred over their Cys-containing homologues if selenium is available. Since no structural information is available for the Vhu and Fru enzymes, we have modelled the large subunit of these enzymes and analyzed the area surrounding the active site. Three [NiFeSe] hydrogenases of the Hys and Vhu types were identified in which the selenocysteine residue is found in a different location in the sequence, which could result in a different coordination to the Ni atom. The high activity and fast reactivation, together with a degree of oxygen tolerance for the H_2-production activity, make the Hys hydrogenases attractive catalysts for technological applications.

Introduction

Hydrogenases (Hases) catalyze the simplest chemical reaction in nature, the reversible oxidation of molecular hydrogen:

$H_2 \rightleftharpoons 2H^+ + 2e^-$

These proteins play a crucial role in the energy metabolism of several microorganisms including bacteria, archaea and lower eukaryotes, which either use hydrogen as an energy source or produce it to dispose of excess reducing equivalents.[1] The Hases are divided into three phylogenetically distinct groups that have a different metal content at the active site: the [NiFe], the [FeFe] and the iron–sulfur cluster-free [Fe] Hases.[2] These proteins, which share no sequence similarity, are a result of convergent evolution since they display some common structural features at the active site, including the presence of carbonyl ligands bound to the iron atom.[2a,3] The [NiFe] and [FeFe] Hases constitute the vast majority of these enzymes, and both contain a binuclear metal active site. The [FeFe] Hases display high catalytic rates but are extremely sensitive to irreversible inactivation by oxygen in the reduced state, whereas [NiFe] Hases show much less sensitivity and can generally recover from this type of inactivation.[4] The [NiFe] Hases are the most abundant group of Hases and have a minimum of two subunits: the catalytic subunit that contains the active site and the electron-transferring subunit that contains one or more iron–sulfur 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, while the other two only bind Ni. The Fe atom is in turn coordinated by three small diatomic ligands, two CN^- and one CO.

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 (Table 1).[1,2c,5] Group 1 includes the periplasmic H_2-uptake Hases, which oxidize hydrogen and reduce the quinone

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pool. They are characterized by the presence of a signal peptide in the small subunit, involved in translocation of the two mature subunits to the periplasm. Most group 1 Hases are associated with a third subunit, cytochrome *b*, responsible for membrane anchoring and quinone reduction, with the exception of periplasmic Hases of *Desulfovibrio* spp. and other sulfate reducers in which their redox partner is a soluble low-potential cytochrome *c* group that transfers electrons to membrane-associated complexes.[6] Group 2 Hases are cytoplasmic and include the cyanobacterial uptake enzymes[7] as well as the H_2 sensors that regulate expression of other Hases.[8] The group 3 Hases are cyto-

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Table 1. Classification of [NiFe] Hases (adapted from ref.[1]).

Group	Subgroup	Function	Gene names of Hases discussed in the text
1	–	Periplasmic H_2-uptake Hases	*hys* (Sec), *hyn* (Cys)
2	a	Cyanobacterial uptake hydrogenases	–
	b	H_2-sensing hydrogenases	–
3	a	F_{420}-reducing hydrogenases	*fru* (Sec), *frh* or *frc* (Cys)
	b	Bifunctional NAD(P) hydrogenases	–
	c	F_{420}-nonreducing or methyl viologen reducing hydrogenases (heterodisulfide reductase associated hydrogenases)	v*hu* (Sec), *mvh* or *vhc* (Cys)
	d	Bidirectional NAD(P)-linked hydrogenases	–
4	–	Membrane-bound H_2 evolving hydrogenases	–

plasmic enzymes with additional subunits, which bind soluble cofactors such as NAD(P) and cofactor F420, but also include the heterodisulfide reductase associated Hases (previously known as methyl viologen reducing Hases or F420-non-reducing Hases). This group includes many enzymes from methanogens,[2b] but some enzymes are also present in bacteria. They are called bidirectional because they may function reversibly under physiological conditions. Group 4 is formed by energy-converting membrane-bound Hases, which are multimeric protein complexes with several transmembrane subunits.[9] These proteins are involved in energy conservation, and their subunits are closely related to subunits of complex I.[10]

In some Hases of the [NiFe] family, named [NiFeSe] Hases, one the cysteine groups coordinating the Ni atom is replaced by a selenocysteine (Sec), as observed in other selenoproteins. The Sec amino acid is structurally identical to Cys, with a selenium atom replacing the sulfur atom. It is designated as the 21st genetically encoded amino acid because it is co-translationally inserted into proteins at an in-frame UGA codon.[11] The recoding of UGA from a stop to a Sec codon requires the presence of a stem-loop structure in the mRNA, named a selenocysteine insertion sequence (SECIS), as well as the presence of a complex cellular machinery known as the selenosome.[12] Sec usage is found in the three domains of life, which suggests its presence in the last universal common ancestor. However, many organisms do not use Sec, and for all (but one) classes of selenoproteins, the homologous Cys-containing proteins are present in the same or in similar organisms. Prokaryotic selenoproteins are mostly involved in redox reactions and typically display higher catalytic activities than their Cys-containing homologues.[12a,13] It is not completely clear what the specific properties of selenium that confer this increase in activity are,[14] but the most relevant properties are likely to be the higher nucleophilicity of selenium, the lower redox potential of Sec-containing redox couples and the increased acidity of Sec, which allows selenols to be active at lower pHs.

A genomic analysis reveals that about 25% of sequenced bacteria utilize Sec and have from 1 to 39 selenoproteins – the most selenoprotein-rich organisms are Deltaproteobacteria and Clostridia.[15] This contrasts with only six identified Sec-utilizing archaeal organisms (13%), belonging to Methanococcales and Methanopyrales orders.[15a,16] The utilization of Sec correlates with organisms favouring anaerobic and thermophilic conditions,[15b] and only a few microorganisms are absolutely dependent on the availability of selenium, with the majority being able to express Cys-containing homologues of their selenoproteins. Despite the high energetic cost of selenoprotein biosynthesis, there is a low exchange rate between Sec and Cys in evolution.[15b,17] The most common event is Cys-to-Sec replacement, which is balanced in evolutionary terms with the loss of selenoprotein genes.[15b,18] 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.

In this work, we review the properties of the [NiFeSe] Hases, which have emerged as attractive catalysts for biotechnological applications, because of their high catalytic activities, in particular, a high hydrogen production activity that is tolerant to moderate amounts of O_2.

Distribution

So far, only a few [NiFeSe] Hases have been isolated, namely those from the sulfate reducers *Desulfovibrio salexigens*,[19] *Dm. baculatum*[20] and *Desulfovibrio vulgaris* Hildenborough,[21] which are group 1 uptake Hases, and from the methanogens *Methanococcus vannielii*[22] and *Methanococcus voltae*,[23] which are group 3 cytoplasmic Hases.

We performed a survey of [NiFeSe] Hase sequences currently present in databases (September 2010). As a starting point we used the database of Trace Element Utilization (dbTEU).[24] In this database, [NiFeSe] Hases are generally annotated as FrhA without distinction of different groups. Some well-characterized Hases such as the one from *Dm. baculatum* are absent from this database, and it was necessary to cross the information retrieved with information from other databases such as UniProt. Finally, the BLAST algorithm was used to search for homologues of the previously found [NiFeSe] Hases. The presence of Sec was manually checked in doubtful cases, and it was found that some Hases annotated as being [NiFeSe] Hases are in fact [NiFe] Hases (e.g. the periplasmic Hase from *Desulfotalea psycrophila*; see below). All the new [NiFeSe] Hases found are present in organisms belonging to one of four classes: the Deltaproteobacteria or Clostridia from Bacteria, and

Methanococci or Methanopyrus from Archaea (limited to genera *Methanococcus, Methanocaldococcus* and *Methanopyrus*). The single exception is the Hase from *Thermodesulfovibrio yellowstonii*, which is a deep branching bacterium from the Nitrospira class. Sequence analysis reveals that all the new [NiFeSe] Hases identified also belong to groups 1 and 3 (Figure 1). The group 1 [NiFeSe] Hases, designated Hys, are periplasmic or membrane-associated-

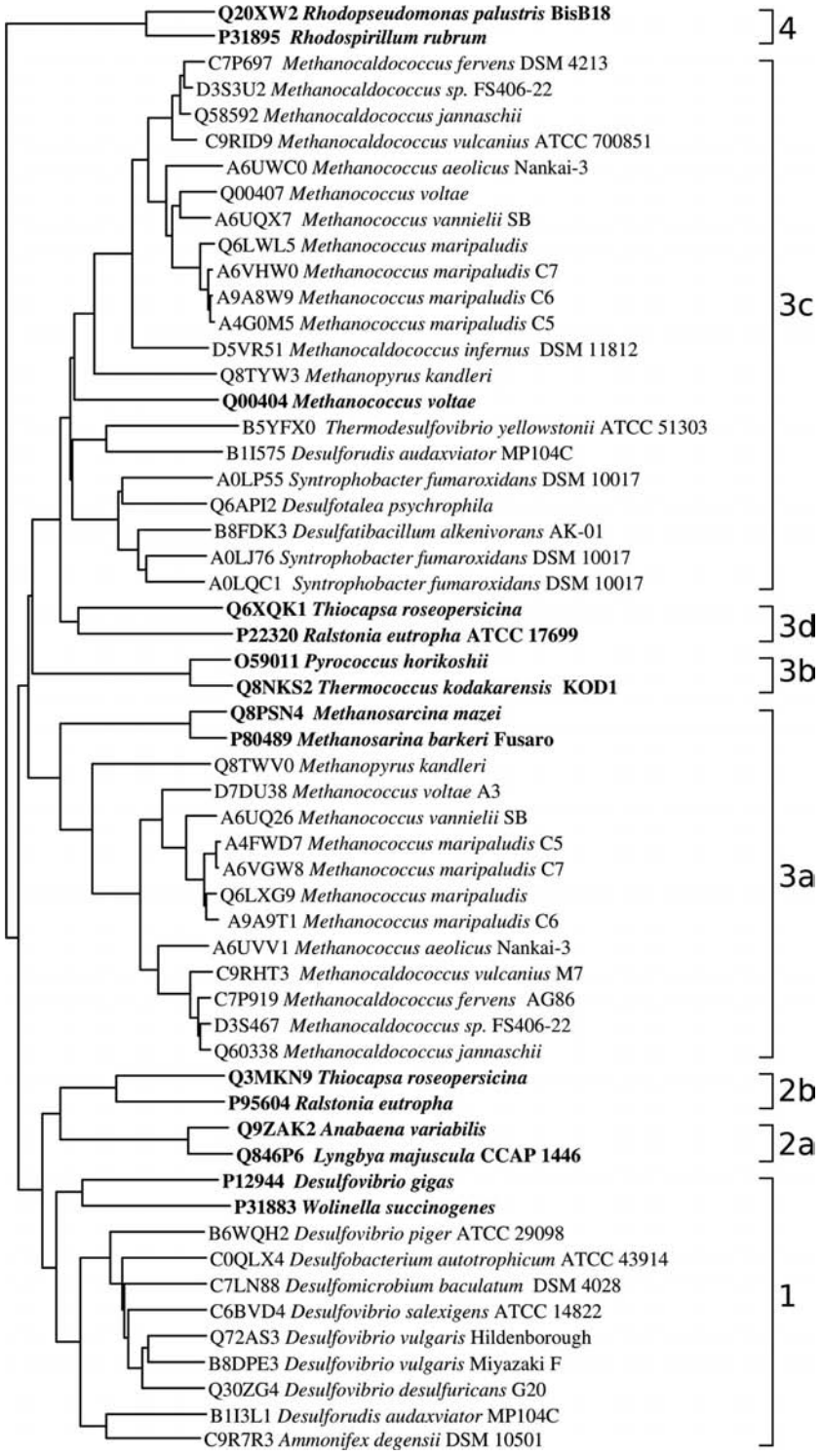


Figure 1. Dendogram of the catalytic subunits of [NiFeSe] Hases. [NiFe] Hases representative of each subgroup are included as reference and highlighted in bold. The numbers on the right represent the different Hase groups. The Uniprot access code of each protein is included before the name of the organism. For the Vhu Hases, the sequences of VhuA and VhuU were concatenated. The multiple sequence alignments were made with ClustalW2.[84] The tree diagram was also calculated with ClustalW2[84] by using the neighbour-joining method and printed with NJPLOT.[85]

uptake Hases that include only two subunits: the large catalytic subunit and the small electron-transfer subunit. Most Hases in this group belong to Deltaproteobacterial organisms, with the exception of the Hases from *Ammonifex degensii* e *Candidatus Desulforudis audaxviator*, which belong to the Clostridia class. This group includes the Hys Hases from *Dm. baculatum* and *D. vulgaris* Hildenborough, which have been investigated more and are the only two [NiFeSe] Hases with a known crystallographic structure.[25] The enzyme from *D. vulgaris* Hildenborough has been shown to be a lipoprotein and thus to associate with the membrane through a lipidic group at the N-terminus of the large subunit.[21,26] It has a truncated lipoprotein signal peptide, restricted to the lipobox, and was the first lipoprotein shown to be translocated through the Tat, rather than the Sec, pathway.[26] The Hys enzyme from *Dm. baculatum* lacks this lipobox and is a soluble protein. Notably, the *D. vulgaris* Hildenborough Hys Hase is more closely related to [NiFe] Hases from other organisms such as *Carboxydothermus hydrogenoformans* and *Desulfotalea psycrophila* than to the [NiFe] Hase from the same organism,[21] which suggests it did not originate from gene duplication but was acquired through lateral gene transfer followed by a Cys-to-Sec replacement. Sequence analysis suggests that these [NiFe] Hases contain three $[4Fe4S]^{2+/1+}$ clusters, which contradicts the idea that all [NiFe] Hases have a mesial $[3Fe4S]^{1+/0}$ cluster.

The remaining [NiFeSe] Hases can be assigned to group 3, the cytoplasmic heteromultimeric bifunctional Hases (Figure 1). Among these Hases, it is possible to distinguish two subgroups: (i) the selenium-containing F_{420}-reducing Hases (Fru) from methanogenic organisms belonging to the genera *Methanococcus*, *Methanocaldoccus* and *Methanopyrus* (the only Sec-utilizing archaea[15a]), which belong to the subgroup 3a; and (ii) the selenium-containing F_{420}-non-reducing Hases (Vhu) present in the same methanogenic organisms and in some bacteria (namely *Syntrophobacter fumaroxidans*, *Ds. psycrophila*, *Candidatus Desulforudis audaxviator* and *T. yellowstonii*), which belong to subgroup 3c. The F_{420}-reducing and the F_{420}-non-reducing (also called methyl viologen reducing) [NiFe] Hases play a key role in the methanogenesis pathway.[2b] The F_{420}-reducing [NiFe] Hases, generally referred to as Frh, reduce the coenzyme F_{420}, which is involved in two reduction steps of methanogenesis from CO_2 and in several anabolic reduction reactions.[2b] The F_{420}-non-reducing [NiFe] Hases, generally referred to as Mvh, are most probably involved in reduction of the heterodisulfide reductase (Hdr), with which they form a tight complex.[27] The Sec-containing homologues of Frh and Mvh are designated as Fru and Vhu, respectively,[28] and the Cys-containing isoenzymes of the Fru and Vhu Hases, present in the *Methanococcus* and the *Methanopyrus* species, are referred to as Frc and Vhc. The *Methanocaldococcus* species do not have the selenium-free Frh and Mvh isozymes.[16]

The Fru Hase is constituted by three subunits: FruA and FruG make up the catalytic heterodimer and the [NiFe] centre is localized in the FruA subunit. The FruB subunit binds the coenzyme F_{420}.[29] The Vhu Hase is constituted by four subunits. The large catalytic protein is divided in two subunits: VhuA and VhuU. VhuA is homologous to the N-terminal portion of the standard MvhA large subunit, and VhuU is homologous to the C-terminal peptide of MvhA. This peptide is proteolytically cleaved during the maturation process and has about 25 amino acid residues only.[23c] Two of the ligands that coordinate the nickel atom belong to VhuA and the other pair of ligands, including Sec, is part of VhuU. These two subunits are encoded by the *vhuA* and *vhuU* genes that probably resulted from a gene-splitting event. The fusion of the *vhuA* and *vhuU* genes has no effect in the activity of the enzyme,[30] and the small fragment is believed to be important in the expression regulation of these Hases.[28] VhuG is the small electron-transferring subunit, and VhuD mediates the electron transfer between VhuG and Hdr.[2b,27]

The Vhu Hases found in bacteria are very similar to those found in the methanogenic archaea, but the catalytic subunit is encoded in a single gene. The three genes encoding the Vhu Hase in bacteria are adjacent to an HdrA homologue, so they may be involved in a similar physiological function as in methanogens, and were most probably acquired through lateral gene transfer from these organisms.[15b] Three of these enzymes are found in *S. fumaroxidans*, which is the known organism encoding the highest number of selenoproteins (39).[15a]

It is noteworthy that all organisms containing [NiFeSe] Hases are either sulfate-reducing bacteria or methanogenic archaea, organisms in which Hases play a key role in energy metabolism. These organisms are present in anaerobic environments, where Sec usage may be an advantage. Indeed, it was found that selenoproteins are more abundant in anaerobic microorganisms.[15]

Activity

The activity of selenoenzymes is greatly increased relative to their Cys-containing homologues,[12a,13] and the same is observed for [NiFeSe] Hases of the Hys, Vhu and Fru families, relative to their corresponding Cys homologues. The Hys enzymes are particularly interesting because they display higher activities for H_2 production than for H_2 oxidation, in solution assays with artificial mediators, which is the reverse of that observed for their Cys homologues (Table 2). Higher ratios of H_2 production to H_2 oxidation in Hys enzymes, relative to standard [NiFe] Hases, have also been reported from electrochemical studies.[31] Two additional important properties were reported for the Hys Hase from *Dm. baculatum*: it shows less inhibition by H_2 than standard [NiFe] Hases, and it can sustain H_2 production in the presence of small quantities of O_2,[31b] which makes this enzyme a very attractive catalyst for biological hydrogen production. Another measure of Hase activity is by their catalysis of H_2 isotope exchange in D_2O or D_2 in H_2O. For example, in the assay of D_2/H_2O medium, the following reactions take place:

Table 2. Catalytic activities of Hases (in U/mg).

Group 1 Hases	H_2 production	H_2 consumption
D. vulgaris Hildenborough HysAB[a] (Sec)	6908	900
Dm. baculatum HysAB[b] (Sec)	2000	–
D. salexigens HysAB[c] (Sec)	1830	1300
Dm. baculatum New Jersey HysAB[d] (Sec)	8600	–
D. gigas HynAB[e] (Cys)	440	1500
D. vulgaris Hildenborough HydAB (FeFe Hase)[e]	4800	50000
Group 3 Hases	**H_2 production**	**H_2 consumption**
M. voltae Vhu[f] (Sec)	–	43540
M. thermoautotrophicum Mvh[g] (Cys)	–	185
M. marburgensis Mvh[h] (Cys)	–	1600
M. voltae Fru[i] (Sec)	–	350
M. thermoautotrophicum Frc[j] (Cys)	–	50

[a] From ref.[21] [b] From ref.[52] [c] From ref.[32a] [d] From ref.[20a] [e] From ref.[82] [f] From ref.[23c] [g] From ref.[34] [h] From ref.[27] [i] From ref.[23b] [j] From ref.[35]

$$\begin{aligned} D_2 + H^+ &\rightleftharpoons HD + D^+ \\ D_2 + 2H^+ &\rightleftharpoons H_2 + 2D^+ \\ HD + H^+ &\rightleftharpoons H_2 + D^+ \end{aligned}$$

The activity is monitored by mass spectrometry by measuring masses 2 (H_2), 3 (HD) and 4 (D_2). 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 Hys Hases is that they catalyze the double isotope exchange of D_2 with protons faster than the single one, contrary to standard [NiFe] Hases.[31a,32] This distinct catalytic behaviour is attributed to the lower pK_a of the active site Sec (5.2) than that of the equivalent cysteine (8.0) in standard [NiFe] Hases.[33] This terminal ligand of the Ni atom is believed to be the base that accepts the proton produced during heterolytic cleavage of dihydrogen at the active site. As a result, the higher acidity of Sec relative to that of Cys should lead to a faster exchange of protons, which thus favours the double-exchange product formation during the isotope-exchange assay, as well as the H_2-production activity.

The heterodisulfide reductase associated Vhu Hase from *M. voltae* has been isolated and displays an extremely high H_2-uptake activity (43540 U/mg)[23c] (Table 2), which can be compared to Cys-homologue Mvh enzymes from other organisms, such as *Methanobacterium thermoautotrophicum* (185 U/mg)[34] or *Methanothermobacter marburgensis* (1600 U/mg).[27] The F_{420}-reducing Hase Fru from *M. voltae* has an activity of 350 U/mg when isolated anaerobically,[23b] but of only 10 U/mg when isolated aerobically.[23a] The Cys-homologue Frc from *Mt. thermoautotrophicum* has an activity of 50 U/mg after aerobic isolation, and it was reported to recover from oxygen inactivation if the enzyme is purified under aerobic conditions and to only suffer irreversible inactivation if exposure to O_2 occurs with the reduced enzyme.[35] The Frc enzyme shows similar activities of F_{420} reduction from H_2 or H_2 production from reduced F_{420}.[36] The latter activity is important, for example, during growth on formate.[37] A common feature of the [NiFeSe] Hases of the Hys, Fru and Vhu types is the presence of three [4Fe4S]$^{2+/1+}$ clusters in the small subunit, rather than a mesial [3Fe4S]$^{1+/0}$ as observed in the more-studied [NiFe] Hases such as that from *D. gigas*. These clusters are responsible for electron transfer with the redox partner. The involvement of a [3Fe4S]$^{1+/0}$ cluster is unexpected because of its high redox potential, but it may not prevent fast electron tunneling as a result of the proximity of the [4Fe4S]$^{2+/1+}$ clusters.[38] To address this question, a mutant of the *D. fructosovorans* [NiFe] Hase with a [4Fe4S]$^{2+/1+}$ cluster instead of a [3Fe4S]$^{1+/0}$ cluster was constructed.[39] 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 the results observed for a mutant of the *M. voltae* Fru Hase containing a [3Fe4S]$^{1+/0}$ cluster instead of a [4Fe4S]$^{2+/1+}$ cluster, which reduced F_{420} with a ten-fold lower rate. This indicates that the low redox potential of the [4Fe4S]$^{2+/1+}$ cluster is important for efficient electron transfer with its redox partner.

Regulation

With some exceptions (e.g. *Methanocaldococcus jannaschi*, which only has genes for a seleno-containing Vhu Hase and is thus dependent on selenium[40]), most organisms that contain [NiFeSe] Hases also contain the corresponding Cys homologues, which likely serve as a back-up system to be used under selenium deprivation.

The regulation of Sec-containing Hases has been investigated in most detail in *M. voltae*,[28] an organism that grows more slowly when selenium is not available.[41] This organism contains two pairs of homologous group 3 [NiFe] Hases, the F_{420}-reducing Hases (Frc and Fru) and the heterodisulfide-associated Hases (Vhc and Vhu). In each pair, one of the enzymes contains Sec as a ligand to the Ni atom (Fru and Vhu), and the other a Cys group in the corresponding position (Frc and Vhc). The transcription of the *frc* and *vhc* genes is coordinately regulated, and occurs only under selenium deprivation, whereas the *fru* and *vhu* genes are transcribed both in the presence and absence of selenium.[42] The *vhc* and *frc* genes are linked by a common

intergenic region that contains a common negative regulatory *cis*-element, as well as two activator elements that are specific for each of the two transcription units.[43] A LysR-type regulator, named HrsM binds to the negative regulatory element when selenium is available, which prevents transcription.[44] A knock-out mutant in the *hrsM* gene led to a derepression of the *vhc* and *frc* promoters. In *Methanococcus maripaludis*, a mutant of the *selB* gene encoding the translation factor specialized for Sec insertion did not show repression of the Sec-independent enzymes in the presence of Se, which reveals that it is not selenium itself that is involved in regulation, but a molecule derived from it such as a selenoprotein or a selenocysteyl-tRNA.[45]

Regulation of group 1 Hys Hases has only been investigated for *D. vulgaris* Hildenborough. Besides Hys, this organism contains two Cys homologues, Hyn1 and Hyn2, as well as a periplasmic [FeFe] Hase, Hyd. The *hys* and *hyn1* genes are adjacent in the genome, which suggests a coordinated regulation.[21] In contrast to *M. voltae*, growth of *D. vulgaris* Hildenborough is not affected by the presence or absence of selenium.[46] However, when selenium is available, there is a strong downregulation of both Hyn and Hyd Hases and a strong increase in the level of the Hys Hase.[46] The situation is similar, but not identical, to that in *M. voltae* and *M. maripaludis*, since the selenium-free Hases are not completely repressed in the presence of Se. In addition, nickel also leads to a downregulation of the Hyd Hase. Thus, by having three types of periplasmic Hases, *D. vulgaris* Hildenborough is equipped to deal with deprivation of both Ni and Se. The Hyn [NiFe] Hase is preferred over the Hyd [FeFe] Hase if Ni is available, and the Hys [NiFeSe] Hase is preferred over the other two in the presence of Se and Ni. However, other factors such as the hydrogen concentration also affect the expression of the three Hases in *D. vulgaris* Hildenborough.[47]

Spectroscopy

The UV/Vis spectra of [NiFeSe] Hases show the expected absorption band at approximately 400 nm for the iron–sulfur clusters.[19,20b] Many EPR studies of [NiFeSe] Hases have been performed since the mid-eighties in order to detect these iron–sulfur clusters and the Ni atom in their paramagnetic states. It has been shown that aerobically oxidized Hys Hases are EPR-silent, which suggests that the these enzymes do not have the $[3Fe4S]^{1+/0}$ cluster present in most H_2-uptake [Ni-Fe] Hases.[19,21,48] Mössbauer and EPR evidence suggest that only two $[4Fe4S]^{2+/1+}$ clusters are present in the *Dm. baculatum* enzyme.[49] However, the crystallographic structures of the Hys Hases from *Dm. baculatum*[25a] and *D. vulgaris* Hildenborough[25b] confirm the absence of a $[3Fe4S]^{1+/0}$ cluster and show that three $[4Fe4S]^{2+/1+}$ clusters are present in the small subunit.

The absence of Ni^{3+} signals in the EPR spectra of the oxidized Hys 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 in oxidized [NiFeSe] Hases with their fast activation upon reduction.[20b,48b] Multifield saturation magnetization data confirm that oxidized *Dm. baculatum* Hase is in a diamagnetic low-spin Ni^{2+} state.[50] In contrast, Sorgenfrei et al. reported that an EPR signal similar to the Ni-B signal of [NiFe] Hases was detected upon air oxidation of the selenium-containing Fru and Vhu Hases from *M. voltae*, although in this the work the amount of paramagnetic Ni per mol of enzyme was not quantified.[23b]

Upon partial reduction of [NiFeSe] Hases with H_2, a rhombic EPR signal can be assigned to a Ni^{3+} state that is reminiscent of the Ni-C state of [NiFe] Hases, in which a hydride bridges both metals of the active site.[51] Table 3 shows the paramagnetic g values measured for different [NiFeSe] Hases for this state. There are few differences between the [NiFeSe] Hases isolated from different microorganisms, which suggests that the structure of their bimetallic centre in the reduced active state is almost the same. Redox titration of the Ni-C state was performed for the *Dm. baculatum* Hase. The formal redox potential measured is –300 mV at pH 8.0.[20b] EPR studies with ^{77}Se-enriched Hases from *Dm. baculatum*[52] and *M. voltae* Vhu[53] and Fru[23b] show hyperfine splitting of the Ni-C signal because of magnetic interaction with the spin of the ^{77}Se nucleus ($I = 1/2$), which indicates the existence of a Se–Ni bond and that the unpaired electronic spin of Ni^{3+} is partially delocalized on the Se atom. These results demonstrated that Sec is coordinated to the Ni site before any crystallographic structure of a [NiFeSe] Hase had been obtained. In contrast, hyperfine splitting of the Ni-C signal because of the ^{1}H nucleus is considerably smaller, which leads only to a slight broadening of the signal in H_2O relative to D_2O.[53b,54]

Table 3. Overview of *g* values of the Ni-C EPR signal for different [NiFeSe] Hases.

Species	*D. salexigens*[a]	*Dm. baculatum*[b]	*M. voltae*[c]	*D. vulgaris*[d]
g_x	2.22	2.22	2.21	2.22
g_y	2.16	2.17	2.15	2.16
g_z	2.00	2.00	2.01	2.00

[a] From ref.[19] [b] From ref.[20b] [c] From ref.[53b] [d] From ref.[21]

Müller et al. compared the distribution of protons and unpaired electrons in the active site of the *Dm. baculatum* [NiFeSe] Hase with the *D. gigas* [NiFe] Hase by high-resolution ^{1}H electron nuclear double resonance (ENDOR) spectroscopy. The authors concluded in this work that only two proton ENDOR signals, assigned to the β-methylene hydrogen atoms of the terminal Cys ligand of the Ni atom, were different between the two Hases (in *Dm. baculatum* Hase the Cys ligand is replaced by a Sec ligand).[54] Thus, this result suggests that the structure of the bimetallic Ni–Fe centre in its active state is not very different in both types of Hases, with the exception of the change in a S atom by a Se atom.

Upon illumination of Hases in the Ni-C state at temperatures of about 30 K, a new EPR signal appears that has been named Ni-L and is assigned to a Ni^{1+} state in which

a proton has photodissociated from the active site, presumably from the bridging hydride ligand.[53b,55] Another paramagnetic state of the active site of a [NiFeSe] Hase was detected when the enzyme was incubated under a 10% CO/90% H_2 atmosphere at 0 °C. This EPR signal is assigned to an active-site state in which the extrinsic CO group is bound to the Ni atom at a position opposite to that of the Sec ligand because the nuclear spins of both ^{77}Se and ^{13}CO interact with the unpaired electron of the Ni atom.[53a] The EPR spectra of the reduced [NiFeSe] Hases at temperatures lower than 20 K yield signals assigned to $[4Fe4S]^{2+/1+}$ clusters, which are fast-relaxing species that are not visible at higher temperatures.[19,20b,21,48a] Interaction of the spin of the proximal $[4Fe4S]^{2+/1+}$ cluster with the spin of Ni^{3+} leads to a splitting of the Ni-C signal below 20 K, as shown in Figure 2 for the reduced *D. vulgaris* Hildenborough [NiFeSe] Hase.

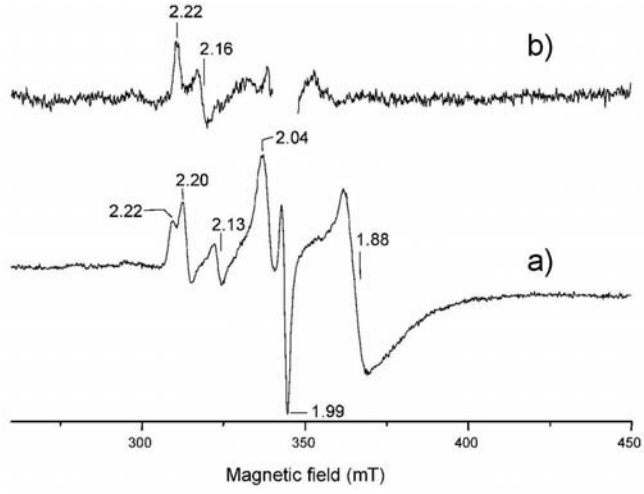


Figure 2. EPR spectra of the H_2-reduced [NiFeSe] Hase from *D. vulgaris* Hildenborough. (a) T = 6.5 K; (b) T = 20 K. Microwave frequency 9.64 GHz, microwave power 2.4 mW, modulation amplitude 1 mT. Reprinted from ref.[21] with permission.

FTIR spectroscopy has proven to be a powerful tool for characterization of the active site of all types of Hases because the CO and CN^- ligands give intense bands, with frequency values highly sensitive to changes in the electronic structure of the active site.[56] Wang et al. reported the first two FTIR spectra of a [NiFeSe] Hase, the as-isolated and the H_2-reduced enzyme forms of *Dm. baculatum*.[57] Both spectra are quite complex because a large number of CO and CN^- peaks are present, which suggests heterogeneity of the samples and makes assignments difficult.

A complete characterization by infrared spectroscopy of the [NiFeSe] Hase from *D. vulgaris* Hildenborough in its different redox states has been reported by De Lacey et al.[58] Two conformations of the active site are detected by FTIR for each redox state, and each conformation has a set of one CO band and two CN^- bands, as shown in Figure 3. Two different EPR-silent, air-stable redox states that are not in equilibrium were identified. One of these states corresponds to the as-isolated enzyme upon aerobic purification (Ni-IS), whereas the other corresponds to the reoxidized enzyme after reduction with H_2 or other reductants (Ni-OX). Upon reduction of these states, the catalytically active states, Ni-C, which is the paramagnetic state detected by EPR, and Ni-R, which is a completely reduced EPR-silent state, appear immediately. 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.[58] Scheme 1 shows a summary of the redox steps between the different active site states of the [NiFeSe] Hase from *D. vulgaris* Hildenborough, as deduced from the FTIR results.

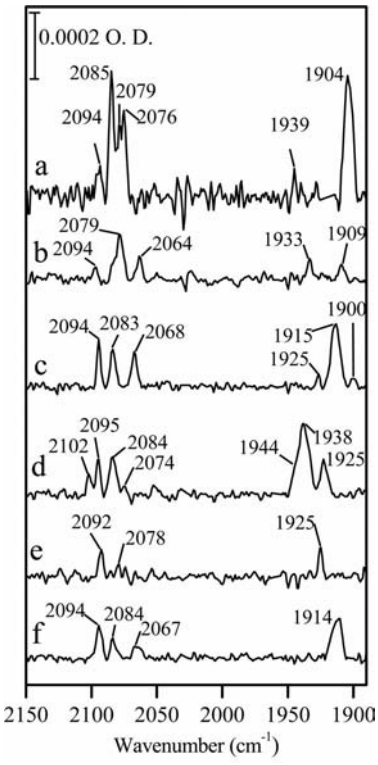
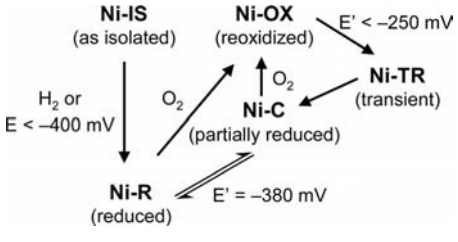


Figure 3. FTIR spectra of 1.0 mM [NiFeSe] Hase from *D. vulgaris* Hildenborough poised in an spectroelectrochemical cell under different redox conditions in the following order: (a) as isolated, open circuit potential +300 mV (Ni-IS); (b) reduced at an applied potential of –435 mV (Ni-R); (c) reoxidized 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. Reprinted from ref.[58] with permission.



Scheme 1. Diagram of the different redox states of *D. vulgaris* Hildenborough [NiFeSe] Hase (adapted from ref.[58]).

The binding of the CO inhibitor to the active site of this Hase has also been studied by FTIR spectroscopy. The results show that the extrinsic CO ligand binds to Ni in both conformations of the active site in a way similar to that in standard [NiFe] Hases, although in one of the CO-inhibited conformations, the active site of the [NiFeSe] Hase is more protected against attack by O_2.[31a] This is in agreement with the above-mentioned Ni-CO EPR signal, which also suggests direct binding of the extrinsic CO ligand to the Ni atom, although in this case, the FTIR and the EPR spectra do not correspond to the same redox state of the active site. The former probably corresponds to a Ni^{2+}-CO state,[31a] whereas the latter is assigned to a Ni^{+1}-CO state.[53a] A few articles have reported on the spectroscopic studies of [NiFeSe] Hases by other techniques besides EPR and FTIR. A Mössbauer spectroscopy study of a ^{57}Fe-enriched sample of [NiFeSe] Hase from *Dsesulfomicrobium norvegicum* by Bell et al. concludes that the iron in this enzyme is predominantly in the $[4Fe4S]^{2+/1+}$ clusters. The clusters are in the diamagnetic $[4Fe4S]^{2+}$ state when the enzyme is oxidized, and are 50% reduced to the paramagnetic state when the enzyme is treated with methyl viologen and sodium dithionite.[59]

These results are in agreement with the EPR data reported for the [NiFeSe] Hases discussed above. A Ni and Se X-ray absorption spectroscopy (XAS) study of the oxidized *Dm. baculatum* [NiFeSe] Hase was performed by Eidness et al. The results were interpreted in terms of a Ni-coordinated Sec, in agreement with the EPR data, with a Ni–Se bond length of 2.4 Å. In addition, a penta- or hexacoordinate Ni site is proposed for the active site in the oxidized state.[60] Wang et al. reported the Ni L-edge soft XAS of *Dm. baculatum* [NiFeSe] Hase in the completely reduced state (Ni-R)[57] and concluded that the most probable electronic configuration of the Ni atom in that state is high-spin Ni^{2+}.

Structure

The first X-ray crystallographic structure of a nickel-containing Hase was obtained in 1995, when Volbeda et al.[61] described the structure of *D. gigas* [NiFe] Hase at a resolution of 2.8 Å. The three-dimensional structure revealed the presence of an iron atom in addition to nickel in the active site, and eleven iron atoms arranged in a chain of three iron–sulfur clusters: two $[4Fe4S]^{2+/1+}$ clusters at the ends and the middle one is a $[3Fe4S]^{1+/0}$ cluster. In addition, the iron centre is bound by three diatomic ligands (one CO and two CN^-) as previously identified by FTIR.[62]

The first X-ray crystal structure of a [NiFeSe] Hase, the periplasmic Hys enzyme from *Dm. baculatum* in its fully reduced and active form, was published in 1999 at a resolution of 2.15 Å.[25a] This structure confirms that the Ni atom has a Sec ligand and reveals that the oxo-bridging ligand hitherto observed in oxidized [NiFe] Hases is absent in the reduced state. It also shows that the small subunit includes three $[4Fe4S]^{2+/1+}$ clusters, and not two as predicted spectroscopically – the $[3Fe4S]^{1+/0}$ mesial cluster of standard [NiFe] Hases (see below) is a $[4Fe4S]^{2+/1+}$ cluster. The second three-dimensional structure of a Sec-containing Hase was published only 11 years later, that of the soluble form of Hys Hase from *D. vulgaris* Hildenborough.[25b] 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, which provides definitive evidence that the oxidized species of the Hys Hases are distinct from those of standard [NiFe] Hases.

These are the only crystal structures available to date of the [NiFeSe] Hases. In both cases, the domain structure of the large and small subunits is typical of group 1 uptake [NiFe] Hases[6a,25] (Figure 4). The coordination scheme of

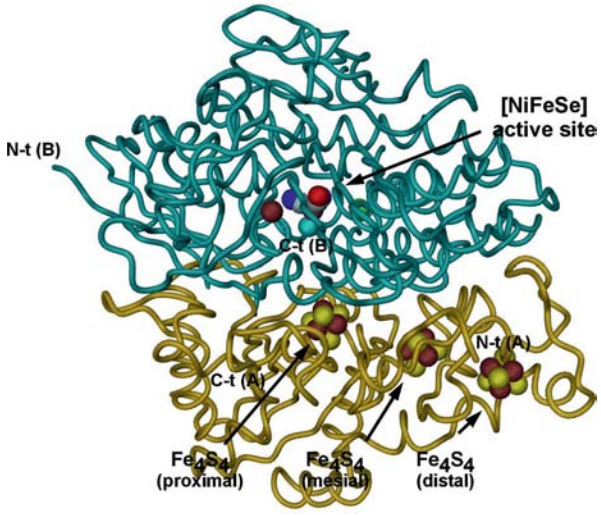
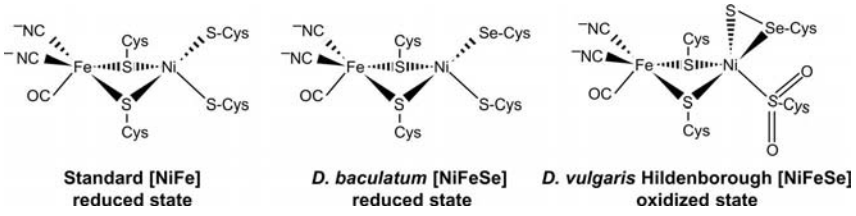


Figure 4. The structure of *D. vulgaris* Hildenborough [NiFeSe] Hase. C^{α} tube diagram representation of the overall structure, with the cofactors represented as spheres; the small subunit (chain A) is gold and the large subunit (chain B) is dark cyan. Atom colours are light blue for carbon, blue for nitrogen, red for oxygen, yellow for sulfur, brown for iron, cyan for nickel and green for Cl^-. Figure prepared with DINO (http://www.dino3d.org).



Scheme 2. Diagram of the active sites in [NiFe] and in [NiFeSe] Hases.

the active site (Scheme 2) and of the $[4Fe4S]^{2+/1+}$ clusters is identical to that described in the [NiFe] Hases from sulfate-reducing bacteria, with the notable difference that the mesial iron–sulfur cluster is an $[4Fe4S]^{2+/1+}$ centre, as opposed to a $[3Fe4S]^{1+/0}$ cluster in the [NiFe] Hases.[61,63] The presence of three $[4Fe4S]^{2+/1+}$ centres seems to be a conserved feature in [NiFeSe] Hases, as indicated by our sequence analysis, and supported by experimental evidence in both Vhu and Fru from *M. voltae*.[48a]

In the *D. vulgaris* Hildenborough [NiFeSe] Hase structure, an extrinsic sulfur atom bound to the Ni atom is found at the active site with partial occupation. In addition, the Sec group terminally attached to the Ni atom is present in three conformations, as interpreted from the electron density (see Figure 5): In two of them, Sec binds the extrinsic sulfur atom, whereas in the third conformer it does not. Conformer I is likely to correspond to an oxidized state, whereas conformer III is identical to the conformer of the fully reduced state in the *Dm. baculatum* Hase structure.[25a] Conformer II is an intermediate between the other two conformers. The oxidized state of the protein therefore includes an extrinsic sulfur atom bound to the Se and Ni atoms, which results in a Sec conformation that is distinct from that of the reduced state.[25b] Interestingly, the known structures of the oxidized and reduced [NiFe] Hases show that the equivalent Cys residue always adopts the same conformation, regardless of the oxidation state of the enzyme.

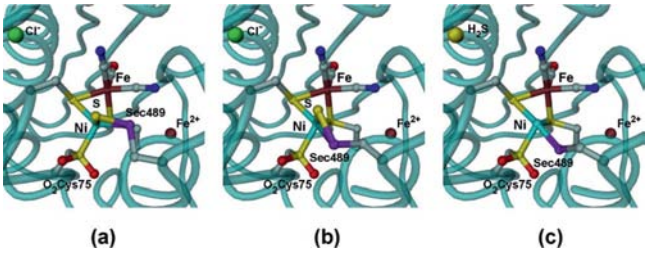


Figure 5. Sec 489 conformers in the active site of *D. vulgaris* Hildenborough [NiFeSe] Hase. C^{α} tube diagram representations of the large subunit, with the $NiFe(CN^{-})_2(CO)$ cluster and the side-chain atoms of the cysteine and selenocysteine residues drawn in ball-and-stick. The colour scheme is the same as in Figure 4, with the selenium atoms in purple. (a) Conformer I, Sec 489 bound to extrinsic sulfur; (b) Conformer II, Sec 489 bound to extrinsic sulfur; (c) Conformer III, Sec 489 with H_2S in the "storage" site, replacing Cl^{-}. Figure prepared with DINO (http://www.dino3d.org).

Several high-resolution crystal structures of [NiFe] Hases from D. *vulgaris* Miyazaki F and *D. fructosovorans*[2a,64] show oxidation of a cysteine at the active site, generally involving a single oxygen atom (sulfenate) at either one of the bridging or terminal cysteines. Surprisingly, in *D. vulgaris* Hildenborough Hys Hase, the terminal cysteine is present in a doubly oxidized state (sulfinate), while still bonded to the Ni atom (2.27 Å) in the active site. This feature has not been reported before in other Hases. The proximal iron–sulfur cluster, located in the small subunit, was also found to be partially oxidized, presumably to $Fe_4S_3O_3$,[25b] a modification which had previously been observed in the three-dimensional structure of the [NiFe] Hase from *D. desulfuricans* ATCC 27774.[65]

In addition to the NiFe active site and the iron–sulfur clusters, the structures of the [NiFeSe] Hases from *Dm. baculatum* and *D. vulgaris* Hildenborough have a Fe ion coordinated by a histidine located at the C-terminus of the large subunit.[25] This histidine residue is highly conserved among several [NiFe] Hases,[1] and the Fe coordination environment is identical to that of the Mg ion found at the equivalent site in [NiFe] Hase structures.[63a,64a,65] A further ligand species is present in the crystal structures of *Dm. baculatum* and *D. vulgaris* Hildenborough: a H_2S molecule and a Cl^{-} ion, respectively, located in similar positions and ca. 7 Å from the active site.[25] This site could represent a storage location for the extrinsic sulfur 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 the oxidized and reduced forms of the enzyme. This is in contrast to the [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 to 2.6 Å in [NiFe] Hase from *D. vulgaris* Myiazaki F[64a]).

The structure of *D. vulgaris* Hildenborough [NiFeSe] Hase provides important evidence with regard to the oxygen tolerance of [NiFeSe] Hases, which allows them to produce H_2 even in the presence of low levels of O_2.[31b] The mechanism of O_2 tolerance has been probed by several studies,[66] namely by measuring H_2 oxidation activity in the presence of O_2 over a range of potentials, pH 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 *D. vulgaris* Hildenborough [NiFeSe] Hase, the Sec conformations in conformers I and II (Figure 5a, b) have the effect of shielding the Ni atom in the active site and blocking its access by other molecules such as O_2. Only in a reduced-like conformation (conformer III, Figure 5c) does the side chain conformation of Sec expose the Ni atom,[25b] as illustrated in Figure 6.

Both [NiFeSe] Hases with known structures, as well as all [NiFe] Hases structurally characterised to date, belong to group 1. Therefore, there is no experimental structural information on [NiFeSe] Hases belonging to the other groups, namely groups 3a and 3b. One way to circumvent this lack of structural information is to use comparative modelling methods.[67] This is possible since there is a significant homology between the members of groups 3a and 3c and the [NiFeSe] Hases from group 1 with known structures. Therefore, we modelled the large subunit structures of FruAG and VhuAUG from *M. voltae*, chosen to represent groups 3a and 3c, respectively. Both large subunits share a 27% sequence identity with the Hys Hase from *D. vulgaris* Hildenbrough, which is not very high, but enough to derive reasonable models. By using this procedure, together with the known X-ray structures, we cover the full spectrum of the known [NiFeSe] Hases. To build comparative models for the FruAG and VhuAUG Hases, we used the X-ray structures of the Hys Hases from *D. vulgaris* Hildenbrough[25b] and *Dm. baculatum*[25a] and the methods im-

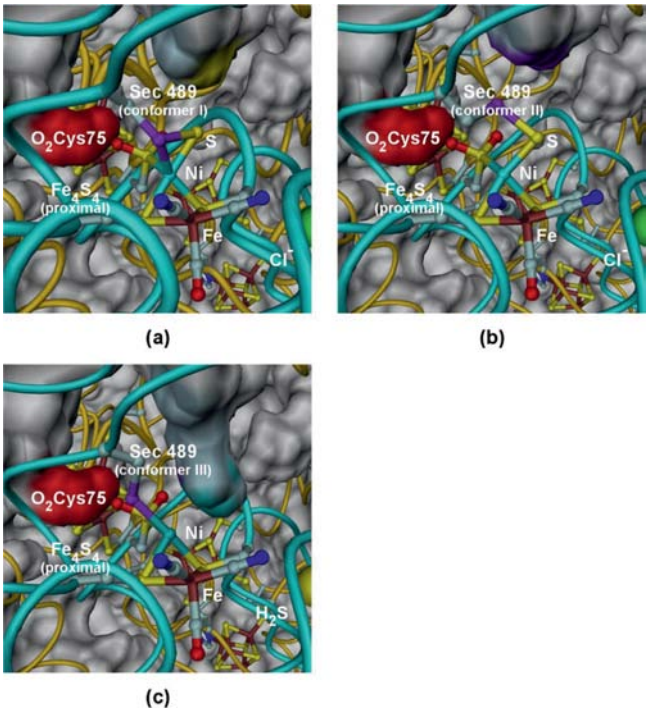


Figure 6. Hydrophobic tunnels in the structure of [NiFeSe] Hase from *D. vulgaris* Hildenborough and its oxygen tolerance. Molecular surface and C^{α} tube diagram representations of the overall structure, with the cofactors represented as ball-and-stick or spheres; the small subunit is gold and the large subunit is cyan. Atom colours are as in Figures 4 and 5. (a) Sec conformer I; (b) Sec conformer II; (c) Sec conformer III. The molecular surfaces were calculated with MSMS[86] by using a probe radius of 1.0 Å and are shown with colours according to their respective contributing active site atoms: purple for selenium, red for oxygen, light blue for carbon and cyan for nickel. Figure prepared with DINO (http://www.dino3d.org).

plemented in MODELLER (version 9.6).[67b] It should be noted that the large subunit of VhuAUG is composed of two peptides, namely VhuA and VhuU.

By comparison of the active sites of the *D. vulgaris* Hildenborough (Figure 7a) and *Dm. baculatum* (Figure 7b) [NiFeSe] Hases with the Vhu (Figure 7c) and Fru models (Figure 7d), it can be observed that Glu28 (*D. vulgaris* Hildenborough sequence numbering), which is thought to play an important role in the catalytic mechanism,[68] is one of the conserved residues in all these enzymes. In addition, a few amino acid residue substitutions are detected around the active site when comparing the Vhu and Fru models with the Hys Hases. In both models, Gly491 near Glu28 was replaced by a serine residue, which may be involved in proton transfer, given its particular position near the conserved glutamate and its H-bond acceptor and donor properties. In the Fru enzyme, Ala81 is substituted by a threonine residue. Some of the residues surrounding the CN^{-}/CO diatomic ligands are also different from those of the group 1 Hases. In the Vhu, Leu82 is replaced by an asparagine residue and Ser443 is replaced by an alanine residue, while in the Fru, only Ser443 is replaced by a valine residue. Overall, the number of aliphatic residues in the vicinity of the active-site metals is higher in the Vhu than in the Hys Hases from *D. vulgaris* Hildenborough and *Dm. baculatum*, but lower in Fru. The substitutions described for Vhu and Fru from *M. voltae* are conserved within the respective Hase group, which suggests that these substitutions are not random events.

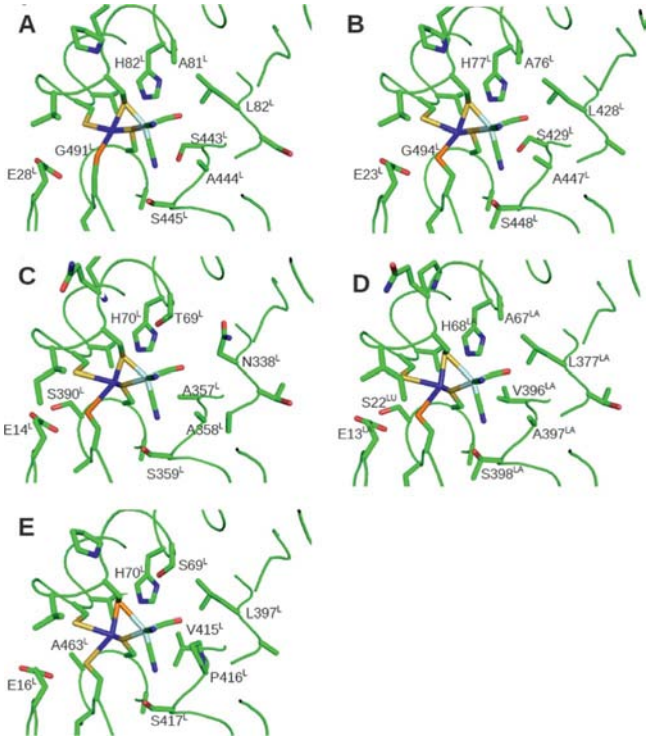


Figure 7. Comparison of the [NiFeSe] active sites and surrounding environments in Hases from different phylogenetic groups. The crystallographic structure of the [NiFeSe] Hases from *D. vulgaris* Hildenborough (A) and *Dm. baculatum* (B), which belong to group 1, were used to derive a model for Fhu from *M. voltae* (C) and for Vhu from *M. voltae* (D), which belong to group 3a and 3c, respectively. The [NiFeSe] Hase from *Candidatus Desulforudis audaxviator* (E) belongs to group 1 and was modelled because it contains the Sec residue in another structural location (in a bridging position). Atom colours are green for carbon, blue for nitrogen, red for oxygen, yellow for sulfur and orange for selenium. Figure prepared with PYMOL (http://www.pymol.org).

In the two Hys Hases with a known structure, and in the majority of the [NiFeSe] Hases, the Sec group is conserved at the terminal position (Figure 7, highlighted in orange), but in the two [NiFeSe] Hases found in *Candidatus Desulforudis audaxviator* and in the [NiFeSe] Hase found in *Thermodesulfovibrio yellowstonii*, the Sec is not at the typical terminal position bound to Ni, but at a bridging position, binding both Ni and Fe atoms. Two of these Hases belong to group 3, and one belongs to group 1. In order to investigate the structural consequences of such a substitution, a model was built for group 1 [NiFeSe] Hase from *Candidatus Desulforudis audaxviator* because it is more similar to the other enzymes with a known structure. The model of this active site can be seen in Figure 7e. The main differences registered in the vicinity of the active site, relative to the [NiFeSe] Hase from *D. vulgaris* Hildenborough, are the sub-

stitution of Ala81 by a serine residue (similar to its substitution by threonine in the enzymes from groups 3a and 3c) and the substitution of Ala444 by a proline residue. Since comparative modelling methods were used, which are conservative in nature, the exact conformation of the segment where this alanine to proline substitution occurs cannot be predicted.

However, we believe that conformational differences are expected here, potentially affecting the protein interaction with the diatomic ligands of the iron atom. Nevertheless, these mutations are not conserved in the other Hases with a Sec group in a bridging position, and consequently their structural significance remains to be elucidated. In the [NiFe] and [NiFeSe] Hases, it is considered that the terminal cysteine residue (equivalent to Sec in [NiFeSe] Hases) is a key player in the mechanism and is responsible for proton transfer, presumably together with Glu28.[2a,65,68a,69] It is thought provoking to see that in this [NiFeSe] Hase, where the Sec group is at another location, there is still structural conservation of a residue equivalent to Glu28. We investigated the presence of protonable residues around the bridging Sec group in the [NiFeSe] Hase from *Candidatus Desulforudis audaxviator* and found no significant differences from the other Hases studied here, which suggests a conservation of the proton transfer mechanism in the whole family of [NiFe] and [NiFeSe] Hases. Therefore, the significance of a Sec group in a bridging position remains obscure.

Mechanistic Studies

In terms of mechanistic studies, both [FeFe] and [NiFe] Hases have attracted the attention of several research groups. These groups have tried to unveil the inner workings of these enzymes, either by targeting the actual quantum mechanical events taking place in the active centre or by trying to understand the fate of the reactants and products from the active site to the surface of the molecules.

Recent quantum mechanics studies targeting the complete reaction mechanisms in [NiFe] Hases have been carried out by the groups of Hall,[69f,70] De Gioia,[71] Hillier[72] and Siegbahn.[69c] The last two reports mentioned present a rather complete study involving the use of QM/MM methods, which include the protein effect on the mechanism. Unfortunately, these studies were not extended to the [NiFeSe] Hases, and, to the best of our knowledge, the only study targeting the active site of these enzymes is by Di Gioia et al.,[73] in which the authors suggest that the S to Se substitution alone cannot explain the catalytic differences between these enzymes and the [NiFe] Hases. Further studies are clearly needed.

In view of the high degree of internalisation of the active site in [NiFe] and [NiFeSe] Hases, the protein structure is an important controlling factor in the access/egress of substrates and products. This was recognised by the authors of the earliest X-ray structure of these enzymes, who described channels for the passage of molecular hydrogen.[61,63b] Molecular hydrogen permeation was initially studied by molecular dynamics simulations of [NiFe] Hase models in implicit solvent[74] and later with complete models using explicit solvent,[75] which evidenced the permeation dynamics and the rate-limiting zones for access to the active site. These same channels also allow the diffusion of inhibitors, such as oxygen and CO. Several experimental studies addressed the role of the residues lining the channels in the control of the molecular hydrogen and oxygen diffusion in [NiFe] Hases,[76] and some mutations have been reported that rendered the enzyme more tolerant towards O_2 inhibition.[76b]The fate of protons was also investigated by using methods based on continuum electrostatics and Monte Carlo simulations[68c] and by quantum chemistry studies.[68b] These works demonstrated the importance of some key groups in proton transfer from the active site to the surface of the molecule. Given the catalytic differences between [NiFe] and [NiFeSe] Hases, it would be interesting to compare both the molecular hydrogen permeation pathways, as well as the proton transfer routes in both systems. Additionally, permeation studies of molecular oxygen would also be interesting, in order to clarify the role of the protein in the different inhibition properties of these two types of Hases.

Another route to study enzyme mechanisms is by the use and development of model compounds, more amenable to mechanistic studies than the large enzymes they try to mimic. Additionally, potential future catalysts with real-world applications are more likely to be small molecules than complete enzymes, in view of their much simpler handling. Several structural analogues for the [NiFe] centre in Hases exist, but this field is much less developed than the extensive work on [FeFe] structural analogues.[77] Unfortunately, to the best of our knowledge, there are no structural analogues of the [NiFeSe] centre. This is clearly an area in need of more studies in order to infer the differences introduced by the presence of a Sec group, which would lead to compounds with possible practical applications.

O_2 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 H_2 productivity in biophotolysis or fermentation, or by the development of biomimetic synthetic catalysts for H_2 production.[4b,78] One of the main problems associated with the application of Hases is their reactivity with oxygen, which leads to inactivation and introduction of oxygen species at the active site.[2a,79] In this respect, the [NiFe] Hases are more favorable because their inactivation is reversible, whereas the reaction of [FeFe] Hases with O_2 leads to irreversible damage.[2a,4b,56,80]

A few [NiFe] Hases isolated from aerobic hydrogen-oxidizing bacteria display a very high tolerance to O_2.[81] However, these [NiFe] Hases have a low activity, particularly in H_2 production because of product inhibition.[66] The Hys [NiFeSe] Hases were initially believed to be oxygen tolerant

because they do not display the long reactivation step observed for typical [NiFe] Hases after exposure to oxygen[20b] or the characteristic inactivated Ni-A and Ni-B species after aerobic isolation.[21,55,82] However, it was recently shown that the Hys enzymes from *Dm. baculatum* and *D. vulgaris* Hildenborough are indeed inactivated by oxygen, but display a very fast reactivation step requiring a low redox potential.[31a,31b] The crystal structure of the oxidized as-isolated *D. vulgaris* Hildenborough Hys Hase showed that no oxide bridging ligand is present at the Ni–Fe site, in contrast to standard [NiFe] Hases, but there are oxygen species bound to a terminal Cys.[25b] The group 3 Vhu and Fru enzymes both form a Ni-B state upon oxidation, which can also be quickly reactivated.[23b]

The quick reactivation of [NiFeSe] Hases together with their high activity indicates that they are interesting targets for biotechnological applications. Hys Hases are particularly attractive catalysts because of the low levels of H_2 inhibition and their high activities, particularly of H_2 production, which were shown to be tolerant to small concentrations of O_2.[31b] These properties were recently explored by adsorbing the *Dm. baculatum* Hys enzyme onto TiO_2 nanoparticles together with a ruthenium photosensitizer, which enabled photocatalytic H_2 production without the need for strict anaerobic conditions.[83] In another application, the Hys enzyme from *D. vulgaris* Hildenborough was bound to a gold electrode allowing direct electron transfer, and moderate electrocatalytic currents for H_2 production were observed.[31c]

Glossary

Sec: selenocysteine

Tat pathway: twin arginine translocation pathway

Sec pathway: general secretory pathway

Hyn: group 1 [NiFe] Hase (HynA, large subunit; Hyn B, small subunit)

Hys: group 1 [NiFeSe] Hase (HysA, large subunit; Hys B, small subunit)

Vhu: F_{420}-non-reducing [NiFeSe] Hases (VhuA, large subunit peptide 1; VhuU, large subunit peptide 2; VhuG, small subunit; VhuD, possible Hdr-interacting subunit)

Fru: F_{420}-reducing [NiFeSe] Hases (FruA, large subunit; FruG, small subunit; FruB, F_{420}-binding subunit)

Mvh or Vhc: F_{420}-non-reducing [NiFe] Hases

Frh or Frc: F_{420}-reducing [NiFe] Hases

Hdr: heterodisulfide reductase

1 U: 1 μmol/min

Acknowledgments

This work was supported by the Fundação para a Ciência e Tecnologia (FCT, MCES, Portugal) and European Union FEDER programs through the research grant PTDC/BIAPRO/70429/2006 and by the Ministerio de Ciencia e Innovacion (Spain) through the grant CTQ2006-12097.

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Received: October 22, 2010
Published Online: January 10, 2011

