ISSN-e: 1856-9811

HYDROGENASE MATURATION ENZYMES

ENZIMAS DE MADURACIÓN DE LA HIDROGENASA

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Abstract:

This work is a part of a comprehensive investigation into hydrogenases, aiming to comprehend their reaction mechanisms, the structure of active sites, and the impact of oxygen on their inactivation. Hydrogenases, enzymes facilitating the reversible reaction of hydrogen production through proton reduction, play a crucial role in energy transformations involving redox reactions. This study explores the sensitivity of hydrogenases to oxygen, elucidating the impact of oxygen concentration on their redox potential and subsequent inactivation due to oxidative stress. Understanding the influence of the environment on the enzyme's activity is pivotal for developing versatile systems.

The research delves into the reaction of oxygen with the H-cluster, an essential component of hydrogenases, yet the specific mechanism remains unknown and subject to debate. This study seeks to uncover the intricacies of this reaction and identify the sites within the H-cluster susceptible to degradation. The overarching goal is to leverage this knowledge for various biotechnological applications, particularly in the development of efficient bioelectrodes for hydrogen production.

Hydrogenases emerge as promising catalysts for hydrogen production due to their high activity and low overpotential. Immobilization experiments on different materials, such as pyrolytic graphite electrodes, are underway, aiming to create devices for biological hydrogen production. The study also explores applications in hydrogen photoelectrochemical production, providing a sustainable alternative to platinum-based catalysts. Immobilizing hydrogenases on various electrodes and coupling them with sensitized nanoparticles has shown promise, with the ultimate ambition of mimicking natural processes to produce hydrogen and oxygen from water in a light-driven water splitting cell.

Key Words: hidrogenases, oxygen sensitivity, redox potential, H-cluster Degradation, enzyme immobilization, Bioelectrodes.

Resumen:

Este trabajo forma parte de una investigación exhaustiva sobre las hidrogenasas, cuyo objetivo es comprender sus mecanismos de reacción, la estructura de los sitios activos y el impacto del oxígeno en su inactivación. Las hidrogenasas, enzimas que facilitan la reacción reversible de producción de hidrógeno mediante la reducción de protones, desempeñan un papel crucial en las transformaciones energéticas que implican reacciones redox. Este estudio explora la sensibilidad de las hidrogenasas al oxígeno, dilucidando el impacto de la concentración de oxígeno sobre su potencial redox y la subsiguiente inactivación debida a estrés oxidativo. Comprender la influencia del entorno en la actividad de la enzima es fundamental para desarrollar sistemas versátiles.

La investigación profundiza en la reacción del oxígeno con el clúster H, un componente esencial de las hidrogenasas, aunque el mecanismo específico sigue siendo desconocido y objeto de debate. Este estudio pretende desvelar los entresijos de esta reacción e identificar los sitios dentro del clúster H susceptibles de degradación. El objetivo global es aprovechar estos conocimientos para diversas aplicaciones biotecnológicas, en particular en el desarrollo de bioelectrodos eficientes para la producción de hidrógeno.

Las hidrogenasas surgen como catalizadores prometedores para la producción de hidrógeno debido a su alta y bajo sobrepotencial. Los experimentos de inmovilización en diferentes materiales, como electrodos de grafito pirolítico, con el objetivo de crear dispositivos para la producción biológica de hidrógeno. El estudio también explora aplicaciones en la fotoelectroquímica del hidrógeno proporcionando una alternativa sostenible a los catalizadores basados en el platino. La inmovilización de hidrogenasas en diversos electrodos y su acoplamiento con nanopartículas sensibilizadas ha resultado prometedora, con la ambición última de imitar los procesos naturales para producir hidrógeno y oxígeno a partir del agua en una célula de división del agua impulsada por la luz.

Palabras clave: hidrogenasas, sensibilidad al oxígeno, potencial redox, degradación del cluster H, inmovilización enzimática, Bioelectrodos.

RECEIVED: 09-03-2023 ACCEPTED: 11-05-2023 PUBLISHED: 15-12-2023

How to quote: Panzone C., (2023). Hydrogenase maturation enzymes. *Anales*, 39, 1-32. https://doi.org/10.58479/acbfn.2023.82

ISSN-e: 2244-8276

INDEX

1. Introduction

This work is part of a bigger project, which involves a basic research about hydrogenases in order to understand the reaction mechanism, the active site structure and how oxygen acts in their inactivation.

Hydrogenases are enzymes able to catalyse the reversible reaction of hydrogen production, through protons reduction. Every reaction involves energy transformations and hydrogenases use chemical energy. In particular, the use of chemical energy involves redox reactions with transfer of electrons and whole atoms of hydrogen. Thus, the reaction of protons reduction that gives hydrogen has to be coupled with a semi-reaction of oxidation, which provides electrons. The couple $2H^+/H_2$ has a reduction potential of -0.42 V and it is coupled with the oxidation of an electron donor, which has a more negative redox potential (in the case of this study methyl viologen was used as electron donor, which has a redox potential of -0.9 V).1

Hydrogenases are very sensitive to oxygen, in fact they are active only in anaerobic conditions. To catalyse the reaction, hydrogenases need electrons at a specific redox potential. Increasing the oxygen concentration leads to the increase of the redox potential and, in these conditions, hydrogenases are not active because oxygen has a more positive redox potential and it is a better acceptor of electrons and because it has a very high oxidative action which involves the degradation of the active site of the hydrogenase. In fact, in presence of very oxidative environments, oxygen is able to form very highly active species (ROS) that attack the active site of the hydrogenase, leading to its degradation. In the hydrogenase, the redox potential of clusters is influenced by the protein structure and it can be regulated by the surroundings. Thus, it is important to understand how the environment can influence the activity of the enzyme, in order to create new systems able to operate at different conditions. Furthermore, it is known that oxygen reacts with the H-cluster but the mechanism is still unknown and very debated. Thus, it is necessary to know how it reacts and which site of the H cluster is involved in the degradation.

The aim of these studies is to manage to use these enzymes in different biotechnological applications. We are interested in studying and using hydrogenases because they are the best catalysts for hydrogen production, with highest activity and low overpotential. Furthermore, they are easy to immobilize, thus they can have many applications in bioelettrodes. Experiments involving the immobilization of hydrogenases via adsorption on different materials are being carried out. For example, the challenge us to create devices for the biological hydrogen production and hydrogen evolution has been observed with hydrogenase absorbed on a pyrolytic graphite electrode.[2] The main application is as hydrogen photoelectrochemical

production catalysts. In fact, so far most of the hydrogen is produced by steam reforming of natural gas but new renewable sources are needed. Water splitting is already used to produce hydrogen from natural sources but this process uses platinum as a catalyst, a very expensive and non-abundant element. Some examples of hydrogenases used as water splitting devices have already been reported. The main advantage of such a system is the use of a less expensive and largely available in nature metallic catalyst, like iron, taking inspiration from natural processes. For example, the hydrogenase was immobilized on a carbon-felt electrode and a porphyrin-sensitized TiO_2 nanoparticle was used as photoanode. A production rate comparable to a platinum electrode was obtained. The ultimate goal is to produce hydrogen and oxygen from water in a light-driven water splitting cell, miming the reaction that occurs in algae and cyanobacteria, coupling the an electrode for O2 evolution containing the isolated PSII with an electrode for H2 evolution containing hydrogenase and the isolated PSI. A big drawback of such a system is the very high oxygen sensitivity of this enzyme.[3]

Hydrogenases can also be used to prevent biocorrosion. In fact, these enzymes can induce the anaerobic microbial induced biocorrosion because they can act as chromate reductases, reducing chromate VI. Thus, they can also be applied in the decontamination of polluted environments.[4]

Hydrogenases can also be used as H_2 biosensors, such as the clay-PBV-hydrogenase electrode reported in the work of Quian et al.[5]. This biosensor was developed with the immobilized hydrogenase sandwiched between two layers of a montmorillonite clay and poly(butylviologen) (PBV) mixture on a glass carbon electrode. Such a system was able to transfer electrons by methyl viologen to the electrode and to catalyse the oxidation of hydrogen to protons.

Hydrogenases can also have application in biofuel cells, exploiting their ability to catalyse the hydrogen oxidation. Fuel cells provide electrical energy from a chemical reaction producing only water as a by-product. They use only H2 and O2 and have higher efficiency than combustion engines. Biofuel cells use enzymes as electrocatalysts, in particular hydrogenases are used at the anode for the hydrogen oxidation. The advantage is that the enzyme is very specific for the substrate and that it is not necessary to separate cathode and anode with a membrane.[3]

Knowing how these enzymes act and their mechanism can lead to the rational design of synthetic mimics that can have large-scale applications. For example, some synthetic analogues of the [Fe-Fe] hydrogenase active site have been shown to be efficient catalysts for oxygen reduction in an aqueous medium.[6] The main issue still remains the low oxygen-tolerance. The development of catalysts that can operate under oxygen presence is the direction investigated by many researchers. Recently, it has been demonstrated that avoiding the inhibiting effects of oxygen with oxygen-tolerant catalysts can be more manageable than creating an oxygen-resistant catalyst. These catalysts are able to reduce oxygen and ROS without being damaged and in this way proton reduction is in competition with O_2 reduction and so hydrogen is produced at lower rate and yield. This problem can be faced designing a catalyst with higher kinetics for proton reduction than for oxygen reduction. The idea is to insert a sacrificial photosystem, which is able to reduce oxygen and to avoid ROS formation, maintaining an high activity into H_2 production. Some systems have been reported as possible

applications for H_2 evolution, using redox active polymers containing viologen moieties that can immobilize the hydrogenase or 3D porous carbon electrodes with the enzyme.[7]

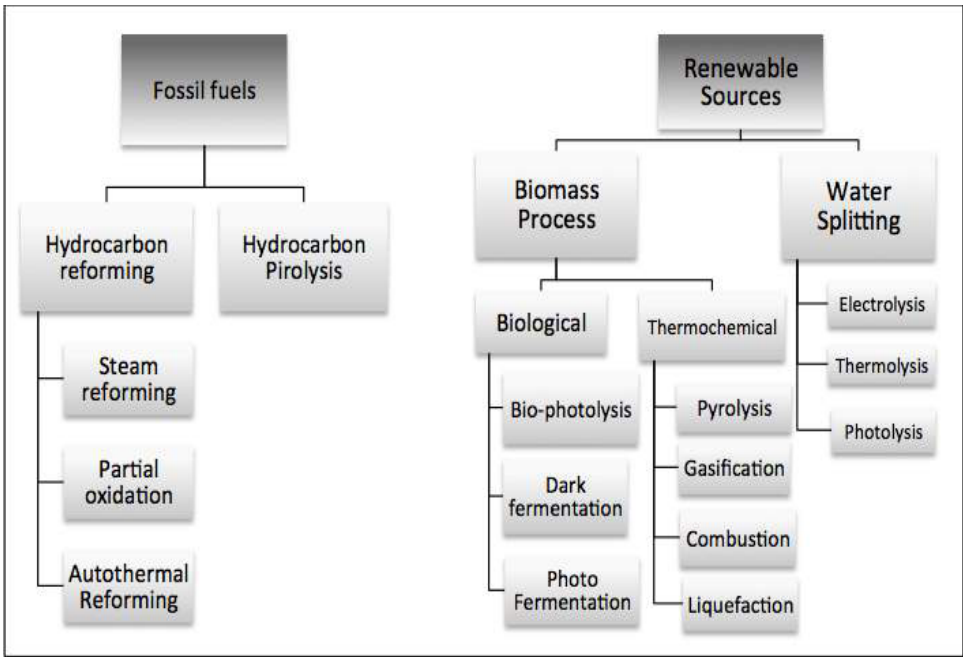
1.1 Hydrogen

The massive use of fossil fuels is starting to pose serious problems to global environment and climate change and this requires the development of new technologies for energy use and storage. The use of renewable energy sources is in expansion but the main problem is the development of storage systems that can store and deliver efficiently the energy when it is required.[8]

There are a variety of renewable sources that could be used for energy production. One promising alternative to fossil fuels is hydrogen. Hydrogen, in fact, is one of the molecules with the highest energy content (higher and lower heating values of hydrogen: 141.9 and 119.9 kJ/g)[9] and it is carbonfree. Moreover, its combustion produces energy and water according to the reaction: $2H_2 + 0_2 \rightarrow 2H_2O$. It can also be safely transported and stored in many ways like compressed gas, cryogenic liquid and solid hydride. However, the use of hydrogen has also its drawbacks, which have to be faced and improved. Among these, molecular hydrogen is not widely available in nature, even if it is very abundant, but it is stored in water or hydrocarbons and it is necessary to efficiently extract it from these compounds.[10] Moreover, hydrogen is nowadays produced through very expensive and polluting methods.

Methods used for hydrogen production are mostly conventional methods, like hydrocarbon reforming and pyrolysis, or renewable processes, using biomass or water as sources (Figure 1.1). Among the hydrocarbon reforming methods, steam reforming, partial oxidation and autothermal reforming are high efficiency processes, widely used for hydrogen production, but they are based on biofuels and produce the greenhouse gas CO_2 as by-product of combustion. Nowadays, the attention is focused on the development of renewable sources based processes. A little quantity of the overall hydrogen is already produced with water electrolysis, which is able to produce very pure hydrogen, without pollutant by-products, but requires a big consumption of electricity. A very successful result would be using photo-catalysis to produce hydrogen from water splitting. Another way to produce hydrogen from renewable sources is biomass processes. A variety of processes exist and they are environmentally benign but the final yields are lower and depend on the raw material used.[8]

Figure 1.1 Hydrogen production methods



Always more technologies are developing using hydrogen as a source of energy, for example fuels cells. These have very promising potential applications because they are able to produce electricity and heat from H_2 and O_2, producing only water as a final product. The main problem in the application of this technology is the lack of molecular hydrogen in nature.

Thus, the necessity of new innovative methods to produce molecular hydrogen at high efficiency and lower cost caused a considerable increase in research in biological hydrogen production. This processes use mostly water or biomass as a source for hydrogen production by bacteria or algae.[8] These microorganisms use their hydrogenase and nitrogenase enzymes to catalyse the reversible reaction of proton reduction: $2H^+ + 2e \leftrightarrow H_2$[11] Thus, our interest into hydrogenases comes from all these reasons.

1.2 Metalloproteins

Metalloprotein is a generic term for a protein containing a metal ion cofactor. It has been estimated that almost 30% of the proteins existing in nature are metalloproteins. The metal ion cofactor is usually coordinated by nitrogen, oxygen or sulphur atoms belonging to amino acids in the polypeptide chain, generally in its side-chains, or by a macrocyclic ligand incorporated into the protein (for example porphyrin, corrin and chlorine). The presence of the metal ion allows the protein to perform its functions that could not easily be performed without it. So, the metal ion is part of the active site and it has a crucial role.

Many efforts have been done to understand the structure and function of these proteins. They have many different functions in cells, such as transport (transferrin for iron), storage (ferritin for iron) or signal transduction. They are also involved in the catalysis of biological processes, such as photosynthesis, respiration, water oxidation, molecular oxygen reduction and nitrogen fixation. Some of them are enzymes and they are called metalloenzymes.[12]

Metalloenzymes are present in all enzyme families and catalyse a wide variety of reactions, so they cannot be associated to a particular group of enzymes, but each metal has been proved to have a specific role in the enzyme.[13] The metal ions that are commonly found in metalloenzymes are iron, zinc and copper, but some proteins also contain nickel, manganese, molybdenum, cobalt, vanadium and calcium. Table 1.1 reports the roles of the most common metal cofactors in enzymes. Some examples are hemoproteins that contain an iron porphyrin as prosthetic group and hydrogenases that can contain iron-sulphur or nickel-iron clusters.

The properties of metalloproteins clearly depend on metal's nature but they can be strictly influenced by many other factors, such as ligands type and coordination number, second coordination sphere on the active site, accessibility of substrate to metal centre and three dimensional structure of the protein.

Table 1.1 Important functions of some metals in algae and bacteria.[13]

Metal	Specific roles in enzymes
K	Protein stabilization
Ca	Protein stabilization
Mg	Protein stabilization
Fe	Oxygen transport, storage and activation, electron transport, superoxide breakdown
Mn	Oxygen evolution, peroxide and superoxide breakdown
Zn	Protein stabilization, hydrolytic cleavage
Cu	Oxygen transport, storage and activation, electron transport, superoxide breakdown
Mo	Oxygen transfer, nitrogen activation
Co	Free radical reactions, nucleophile

Thus, knowing the tri-dimensional structures and the active site configuration is very important in the understanding of these proteins. Techniques for the investigation of chemical and structural properties are available. The most relevant are spectroscopic tools, such as electron paramagnetic resonance (EPR), electron-nuclear double resonance (ENDOR), electron spin resonance (ESR), resonance Raman or Mössbauer spectroscopy. Another powerful technique is X-ray protein crystallography which allows the resolution of the spatial arrangement of the atoms of the proteins.[14]

1.3 Iron-sulfur clusters

Among the metal clusters usually found in proteins, some of the most common are iron-sulfur clusters. Iron is one of the most abundant atoms on earth and it often acts as enzyme cofactor or prosthetic group. Iron can be bound to amino acidic chains directly or via other atoms, such as sulfur in the [Fe-S]-cluster.[15]

[Fe-S]-clusters often have an important role in proteins and were found to be very common in the oldest components of living matter, including Archea, bacteria, plants and animals. They are believed to have contributed to success of life on earth.[16] Studies on this cluster started in 1960s when they were found to be involved in oxidoreductive reactions in many plants and microorganisms. Later, in the 1970s it was shown that iron-sulfur complexes could be artificially synthesised as analogue complexes of the protein active site.[17]

Proteins with [Fe-S]-clusters can take part into a lot of reactions, but they are mainly involved in oxidation-reduction reactions and electron transfer. In fact, iron is able to switch very easily between the Fe3+ oxidized and the Fe2+ reduced forms, behaving as electron donor or acceptor in many biological reactions.[18] For example, iron is involved in cellular respiration in bacteria and mitochondria.5 Another important function of this cluster is that, as they are able to reversibly bind Fe and S, they acts as Fe and S storage for the activation of enzymes or substrates, like in the enzyme aconitase, which has the function to catalyse the reversible transformation of citrate to isocitrate in the citric acid cycle.[19] Moreover, it has been shown that they are involved in DNA replication and repair. For example, DNA polymerase-ε requires the presence of a [Fe-S]-cluster to replicate DNA strands and repair damaged ones.[20]

Different kinds of [Fe-S]-cluster exist and they are generally coordinated to cysteines. The most common conformations are the rhombic arrangement [2Fe-2S], which contains 2 atoms of iron connected to four cysteines via two bridging sulfide ions, and the cubane form [4Fe -4S], that consists in four iron atoms and four sulfur atoms coordinated to four sulfhydryl side chains of cysteines (Figure 1.2). Other clusters have been observed, like the [1Fe-0S], containing one single iron atom coordinated to four cysteines (such as in rubredoxin) and the [3Fe-3S] or more complex form like [7Fe-8S], [8Fe-7S] or [8Fe-8S] (observed in molybdenum-iron proteins).[12]

Studies on the [Fe-S]-cluster assembly process showed that this process occurs *in vivo* with three different machineries: the NIF system, observed the first time in *Azotobacter vinelandii*, for specific maturation of nitrogenase in azototrophic bacteria; the ISC assembly, observed in mitochondria of yeast and other eukaryotes, for the generation of [Fe-S] proteins under normal conditions; and the SUF system, observed in *Erwinia chrysanthemi* and *Escherichia coli* for the generation of [Fe-S] proteins under oxidative-stress conditions. During evolution, the latter two mechanisms were transferred from bacteria to eukaryotes, which were shown to contain [Fe-S]-clusters in mitochondria, cytosol and nucleus. In p articular, mitochondria contain the ISC assembly machinery, while plastids host protein from SUF machinery. It was shown that the entire process was highly conserved from yeast to human. It always requires 1) a sulfur donor (normally a cysteine) that can release sulfur through a cysteine desulphurase, creating a persulphide as an intermediate; 2) a specific iron donor; 3) an electron transfer

(normally performed by bacterial and mitochondrial components), in order to reduce S; 4) a scaffold protein, as platform for the biosynthesis of the [Fe-S] cluster. In fact they can bind [Fe-S]- clusters with labile binds, allowing an easy and stable transfer of the cluster to the target protein; 5) a cluster transfer protein which is needed for the correct cluster transfer to apo-proteins (Figure 1.3).[21]

Figure 1.2 Iron-sulfur clusters commonly found in proteins.

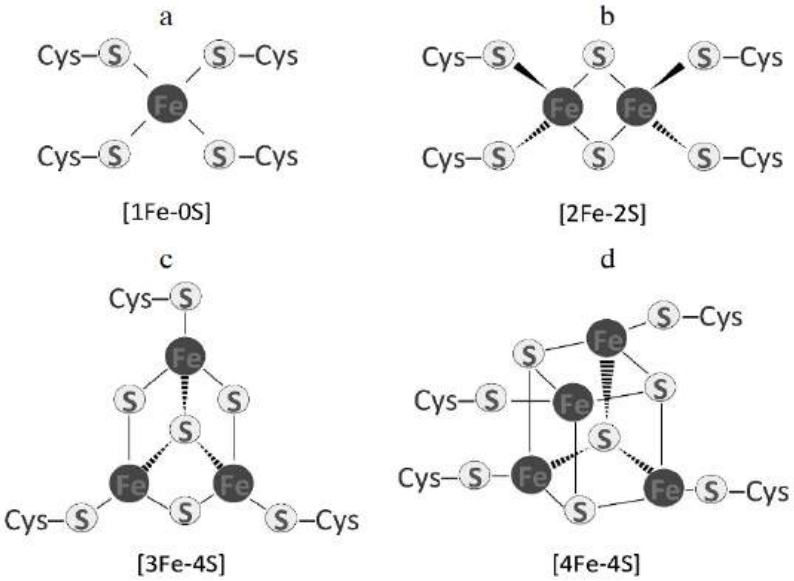
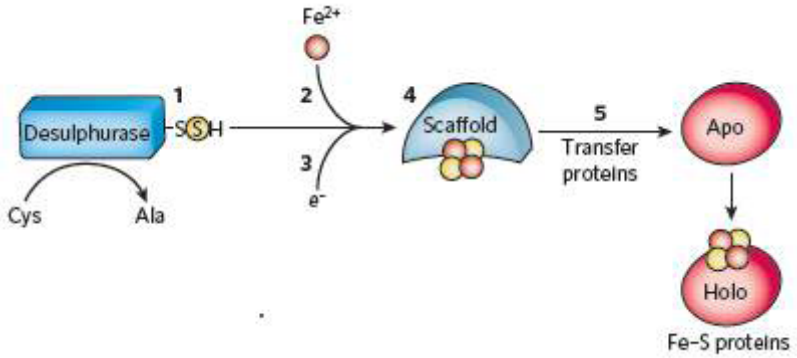


Figure 1.3 Biosynthetic principles of [Fe-S]-protein biogenesis.



The cysteine desulphurase releases sulphur from a Cys, converting it into an Ala. The sulphur obtained is combined with Fe^{2+} and electrons in order to assemble the [Fe-S]-cluster into a scaffold protein. The assembled cluster is then transferred to the apo-protein, converting it into an active holo-protein.[21]

1.4 Hydrogenases

Among iron-sulphur proteins, hydrogenases are a class of protein of special interest. Research on this class is significantly growing, since 2000, due to their inherent applicability in development of renewable energy technologies, based on hydrogen. New methods for producing hydrogen are needed from renewable sources and low cost processes.

Hydrogenases are metalloenzymes that can catalyse the reversible reaction of conversion of dihydrogen into protons and electrons at very high catalytic rates (~10 000s-1)[22]:

$$H2(\rightleftharpoons H + H^{\cdot}) \rightleftharpoons 2H+ + 2e^{-} \quad (2.1)$$

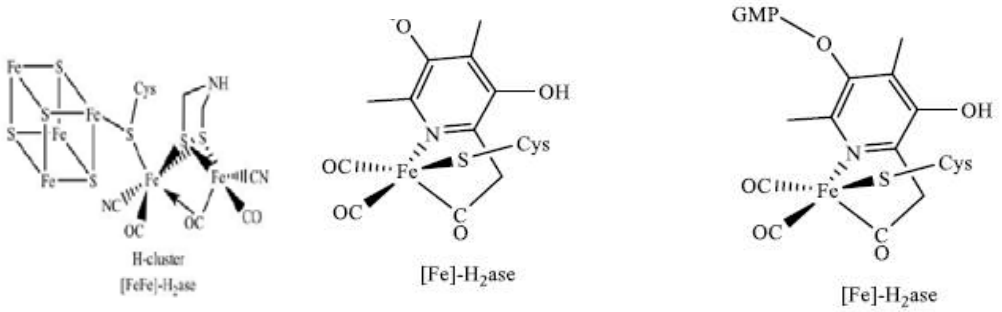
This reaction takes place in presence of an active site that contains a metal, which allows the pH of H2 to dramatically decrease, leading to the heterolytic splitting of the molecule.

Hydrogenases can be classified in three classes: [Ni-Fe]-hydrogenases, [Fe-Fe]-hydrogenases and metal-free hydrogenases. The latter class contains one single iron atom and can activate hydrogen only in presence of another substrate and it is only found in methanogens. The most interesting classes are [Ni-Fe]- and [Fe-Fe]-hydrogenases.[3]

[Ni-Fe]-hydrogenases have been isolated as heterodimers, where the big subunit contains the bimetallic [Ni-Fe] center of the active site, deeply buried in the protein, and the little subunit contains some [Fe-S]-clusters, important for the electrons transfer and essential for the H2 activation. The active site is thus made up of two atoms, one of iron and one of nickel, connected via two cysteines (Figure 1.4).[23] [Ni-Fe]-hydrogenases are involved in hydrogen consumption, while [Fe-Fe]-hydrogenases are normally involved in hydrogen production.

[Fe-Fe]-hydrogenases, normally known as HydA, have an active site made up of a [4Fe-4S]-cluster connected to a [2Fe]-subcluster via a thiolate of a cysteine. This complex is called H-cluster. The cysteine is the only bind between the [4Fe-4S]-cluster and the [2Fe]-subcluster. The two iron atoms of the di-iron subcluster are connected via an azapropanedithiolate bridge. The coordination sphere is filled by a CO and a CN group (Figure 1.4). HydAs differs greatly in size and number of accessory clusters, in fact, depending on the microorganism, HydA can have a single [4Fe-4S]-cluster (the one contained in the H cluster) or additional [4Fe-4S]-clusters that are involved in electron-transfer, from the surface to the active site of the protein.[24]

Figure 1.4 Active sites of the different classes of hydrogenases: H-cluster of [Fe-Fe]-hydrogenase (on the left), [Ni-Fe]-hydrogenase (at the centre) and [Fe]-only hydrogenase (on the right).



1.5 [Fe-Fe]-hydrogenases maturation

To become active, the [Fe-Fe]-hydrogenase polypeptide encoded by the *hydA* gene has to incorporate the [Fe-S]-cluster(s) and the di-iron subcluster. This is called maturation process, which is a very complex post-translational process and involves difficult reactions. In fact, it involves the synthesis of the CO and CN, the dithiolate ligand, the assembly of the diiron subcluster and its incorporation into the [4Fe-4S] protein and the assembly and transfer of the additional [Fe-S]-clusters. The whole process has to be tightly controlled because CN- and CO can be produced at toxic level, as well as hydrolytically sensitive dithiolate.

The pathway that occurs in nature for the hydrogenases maturation is being revealed by a variety of methods and is still partly unknown.

One of the first clues of the maturation process was the identification in the genome of *Chlamydomonas reinhardtii* of genes coding for the three accessory proteins, HydE, HydF and HydG, required for the maturation of an active hydrogenase. It was also shown that many organisms containing [Fe-Fe]-hydrogenases have in their genome genes coding for these three maturases.[25] Moreover, the first example of heterologous expression25 and the first *in vitro* activation26 of an active [Fe-Fe]-hydrogenase revealed that all the three proteins are necessary for the hydrogenase activation. This activation does not require the addition of cluster precursors, meaning that the maturation machinery is able to synthetize and transfer it to HydA.[26] However, the addition of iron, sulphide, SAM (S-adenosyl-L-methonine), cysteine and tyrosine was demonstrated to enhance the hydrogenase activity, suggesting that iron and sulphide reconstitute the iron-sulphur cluster and the SAM added is probably essential for the HydE and HydG proteins.[27] Moreover, it has been shown that the maturation process needs the [4Fe-4S] component of the H-cluster to be assembled before the [2Fe]-subcluster insertion into the protein. Thus, maturation requires the three maturases HydE, HydF and HydG for the synthesis of the [2Fe]-subcluster and its transfer and incorporation into the apo-hydrogenase.[28]

1.5.1 The Hyd protein machinery

Production of an active [Fe-Fe]-hydrogenase requires the simultaneous action of HydA with HydF, HydE and HydG that together compose the so-called HYD machinery.

Two of the additional proteins, HydE and HydG, belong to the radical SAM family. This family of enzymes generally catalyses difficult chemical reactions such as CH to C-S bond formation and glycyl radical formation.[29,30]

HydG contains two [4Fe-4S]-clusters: one is chelated by the typical radical SAM proteins motif CysX3CysX2Cys in the N-terminal domain and the second is chelated by the CysX2CysX22Cys motif in the C-terminal domain. Each cluster has its own role: the N-terminal cluster is required for SAM reductive cleavage and tyrosine cleavage to produce p-cresol, while the C-terminal cluster is required for the production of the diatomic groups CO and CN[31]. It has been shown that both the clusters are essential for activity.[32] Some experiments showed that HydG uses tyrosine as a substrate to synthetize CO and CN-. In fact, Pilet et al. in 2009 demonstrated that HydG was able to cleave tyrosine in pcresol n a SAM-dependent reaction[33] and then, it was shown that HydG produces CN- and CO using tyrosine as substrate.[34,35] Subsequently, Kuchernreuther et al. showed that on a cell-free system both the diatomic groups derive from tyrosine.[36] Furthermore, spectroscopic studied demonstrated that HydG provides both the diatomic ligands (CO and CN-) and the iron for the [2Fe]-cluster, necessary for the formation of the H-cluster.[37] In fact, Stopped-flow FTIR studies provided evidence for the formation on HydG of an iron-bound complex, containing CO- and CN-, the [Fe(CO2 -)CN-] synthon, which is considered to be the effective precursor of the H-cluster. Suess et al. showed that Cys binds the auxiliary iron-sulfur cluster of HydG and suggested that Cys serves as ligand platform where this synthon is built.[38]

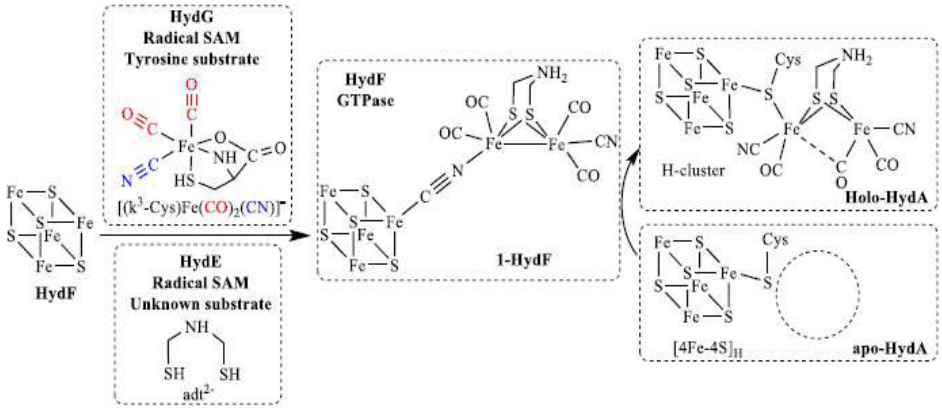
HydE, as HydG, binds iron-sulfur clusters and catalyses the SAM reductive cleavage. Iron analysis, UV-Vis and EPR spectroscopy showed that it contains two [4Fe-4S]-clusters: one is bound to the radical SAM motif CysX3CysX2Cys in the N-terminal domain and is very conserved in HydE; the second seems to be very labile and is presumed not to be involved in H-cluster biosynthesis.[39] X-ray structure of HydE from *Thermotoga maritima* showed that the second cluster is located on the molecular surface and it probably bounds variable amounts of iron and sulphide.[40] Pilet and al.[33] reported that HydE contains three anion-binding sites in a large cavity and one of them can bind SCNwith high affinity. The substrate used by HydE is still unknown but some hypothesis can be made, based on early studies. Kuchernreuther et al.[41] reported that incubation of HydA from *C. reinhardtii* with SAM, cysteine, tyrosine, iron, sulphide and maturases increased 5 fold hydrogenase activity. Given the demonstration that tyrosine is the substrate of HydG[33-36] and SAM is required for radical SAM activity, cysteine reasonably seems to be a possible a substrate for HydE, but this is not been demonstrated yet. Furthermore, Betz et al.[40] suggested that HydE substrate contains a thiol functional group. HydE is presumed to provide the dithiomethylamine bridge of the H-cluster, since it has been demonstrated that HydG provides the iron, CO and CN- components of that cluster.

While the two proteins, HydE and HydG, are part of the radical SAM family, the third protein required for the hydrogenase activation, HydF, is a GTP-ase protein. Spectroscopic studies showed that this protein is able to bind a guanosintriphosphate and contains a [4Fe-4S]-cluster ligated by three cysteines and by a non-cysteinyl ligand, which has been subject of controversy and seems to vary depending on the local residues.[42] It was also observed the presence of an additional [2Fe]-subcluster, ligated by CO and CN-.[43] From size-exclusion chromatography, static light scattering data and the crystal structures[44,45], it was demonstrated that the protein is stable as a dimer where each monomer contains three domains, the GTP binding domain, the dimerization domain and the metal-cluster binding domain. Further information about HydF structure is discussed in Section 2.8.1.

Concerning the role of HydF in the HYD machinery, in 2008 McGlynn and al.[46] postulated that the GTP-ase domain is probably not involved in cluster transfer and that HydF acts as a scaffold or carrier protein in the H-cluster assembly. The hypothesis that HydF has a key role in the H-cluster assembly was encouraged by Czech et al.[43] and, later, it was proposed that a dimeric form of HydF interacts with HydE and HydG in a GTP-bound form and then the GTP is dissociated, in order to allow interactions with HydA.[44] HydF has a key role, being able to act as a carrier and scaffold protein. In fact, this indicates that it is able to interact (i) with HydE and HydG, in order to assemble the binuclear subcluster and (ii) with the apo-hydrogenase, when the [2Fe]-subcluster is transferred to the latter. Interactions between HydF and the other two maturases have been studied by Vallese et al.[47] who showed that (i) HydF can interact with both HydE and HydG, independently of its GTPase properties and (ii) HydE and HydG separately participate in creating structural modifications to HydF, allowing its interaction with the apo-hydrogenase.

The most likely mechanism is that the whole HYD machinery synthesizes and inserts a [2Fe]- subcluster and its ligands into the apo-HydA already containing the [4Fe-4S]-cluster. All the three maturases are essential: HydG carries out the synthesis of the [Fe(CO2)CN-] synthon from tyrosine, HydE is supposed to provide the azadithiolate ligand from un unknown substrate and HydF interacts with the two maturases in order to assemble all the components and to form a diiron complex which is then transferred to apo-hydrogenase, resulting in the activation of the protein (Figure 1.5).

Figure 1.5 Schematic representation of apo-HydA maturation.



HydF interacts with HydG and with HydE. HydG provides the [Fe(CO2)CN-] synthon, using tyrosine as substrate, and HydE probably provides the azadithiolate ligand, from un unknown substrate. The three maturases interacts, HydF assemblees all the components creating a complex, 1-HydF, which contains the diiron subcluster. This complex is transferred to the apo-[Fe-Fe]- hydrogenase which is transformed into an active protein.[48]

Although the mechanism of hydrogenase maturation is being deeply studied, further investigations are required. In fact, many aspects still have to be defined, such as the role of the GTP-ase domain, HydE substrate and especially the order in which the maturases act. Broderick et al.[28] proposed two possible pathways for the [2Fe]-subcluster assembly, which particularly differ in the actions order: in the first one HydE and HydG separately interact with HydF which then transfer the diiron complex to HydA; in the alternate pathway HydG delivers iron bound cyanide and CO to HydF as cyano-carbonyl-Fe units, where azadithiolate addition connects the two mononuclear Fe species.

Another interesting remark is that in *C. reinhardtii* HydE and HydF have been observed to form a single peptide, named HydEF, suggesting that these two maturases are likely to form a complex during maturation process.[49]

1.6 Chemical maturation

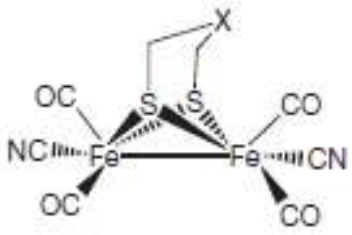
As explained in the previous section, in nature hydrogenases are activated by a very complex mechanism, which involves three maturases and aims at the assembly of the H-cluster. Studies on the possibility to chemically activate hydrogenases are being carried out. Recently, it has been shown that hydrogenases can be chemically activated with a process that does not involve the co-expression of the maturases. In fact, chemically synthesized diiron complexes

can be transferred, through the maturase HydF, to the [Fe-Fe]-hydrogenase, already containing the [4Fe-4S]-cluster. Even if these diiron complexes alone are not active, when inserted in the hydrogenase they showed a high activity, demonstrating the importance of the protein environment.[50]

Basing on the demonstration that anaerobically purified HydA from *C. reinhardtii* (*Cr*HydA1) could be activated by incubation with HydF from *Clostridium acetobutylicum*[43], in 2003, the first example of an artificial maturase system was reported. It was demonstrated that HydF from *T. maritima,* containing the [4Fe-4S]-cluster and expressed in *E. coli,* could incorporate synthetic diiron complexes 1, 2 and 3 (Figure 1.6), leading to new hybrid proteins x-HydF, after a chemical reaction in anaerobic conditions. Spectroscopic characterization, such as FTIR, EPR and HYSCORE, provided evidence of this incorporation. Then, it was shown that only the hybrid 2-HydF protein was able to activate HydA from *C. reinhardtii* containing only the [4Fe-4S]-cluster. So, this work demonstrated that complex 2 can be efficiently transferred from HydF to HydA and is able to activate the apo-hydrogenase, proving that the natural precursor is likely to be very similar to this complex.51 Another step forward was made when it was shown that the synthetic di-iron complexes could be directly incorporated by HydAs.[52] This procedure allows obtaining an active hydrogenase with an activity comparable to the one measured for the wild type. Esselborn et al.[50] showed that the crystal structures of wild type active HydA from *Clostridium pasteurianum* (CpI) and the semisynthetic CpI maturated with complex 2 (CpI^{ADT}) have identical structures.

These results demonstrated that the synthetic complex undergoes some transformations in order to be converted to the natural active substrate. Among these transformations, an isomerisation of the CNligand is needed, as well as a substitution of a CO by a cysteine ligand of the [4Fe-4S]-cluster of HydA.

Figure 1.6 Structures of the synthetic mimics which were tried. X=CH2 in complex 1; X=NH in complex 2; X=O in complex 3.[51]

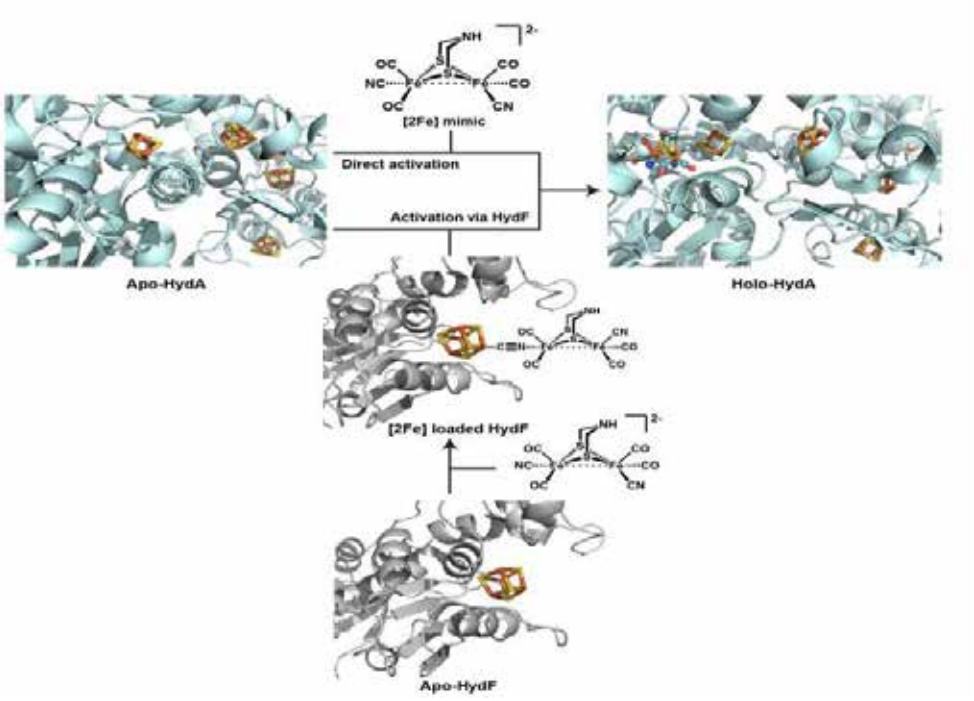


Thus, two possible patterns can be followed for the hydrogenase chemical maturation (Figure 1.7): activation of apo-HydA can be carried out directly with the diiron mimic or via HydF. Both the mechanisms proved that only the complex containing the azadithiolatate bridge was able to obtain HydA with high enzymatic activities, underlining the central role of this nitrogen containing bridge in enzyme activity.

Recently, it has been shown the possibility to avoid not only the use of the HYD machinery but also the bypass of the strictly complete anaerobic purification procedure. In fact, it was shown that it was sufficient to heterologously express HydA from *Megasphaera elsdenii* in *E. coli*, aerobically purify the apo-protein and chemically activate it *in vitro*, in order to obtain an active hydrogenase. The chemical activation requires the anaerobic reconstitution of the [4Fe-4S]-clusters by treatment with iron and sulfur and the anaerobic incorporation of the [2Fe]-subcluster by reaction with the $[Fe2(adt)(CO)4(CN)2]^{2-}$ complex. The effective incorporation of the complex inside MeHydA was proved by FTIR spectroscopy and activity assays.[53]

This synthetic maturation methodology was a very important discovery and helped significantly the identification of the nature of the central atom in the dithiolate bridge of the [2Fe]-subcluster. However, some severe drawbacks still exist, preventing the use of [Fe-Fe]-hydrogenases in biotechnological applications. Among these, the necessity to manipulate these proteins in strictly anaerobic conditions to avoid oxygen mediated inactivation is the biggest obstacle for their large-scale applications.

Figure 1.7 Possible patterns for apo-HydA activation.



The first one consists in activation of apo-HydA via incubation with HydF. The latter is firstly incubated with a diiron complex, which is incorporated in the maturase. Then, [2Fe]-HydF is incubated with apo-HydA and the diiron subcluster is efficiently transferred to the hydrogenase, resulting in an active protein. The second process consists in direct activation of apo-HydA with diiron complex.[62]

1.7 Artificial hydrogenases

As already discussed before, hydrogenases have been widely studied for their potential technological applications, as they rely on non-noble, cheap and abundant metals. However, their use in large-scale applications is still limited by three factors: (i) they are difficult to produce in large quantities, (ii) they are extremely sensitive to oxygen, so they must be manipulated in strictly anaerobic conditions and (iii) it is very difficult to produce them in an active form. This latter has been partly faced with the chemical maturation.

In the last years, a variety of biomimetic and bioinspired metal complexes have been synthetized, allowing the reproduction of structures and functions of the natural metal sites through simple and low-molecular weight synthetic organometallic compounds. For example, many complexes have been synthetized as models for the active site of [NiFe]-hydrogenases. Among these, it was demonstrated that the heteronuclear Ni/Fe[54,55], Ni/Ru56 and Ni/Mn57 complexes showed catalytic activity for proton reduction.

However, none of these compounds showed an activity comparable to the one of hydrogenases, probably because of a lack of interactions with polypeptide chains. Thus, it was considered the idea to improve the performances of the mimics, incorporating them into the protein and designing an artificial hydrogenase.[58]

The concept of artificial enzymes has its origins in the work of Wilson and Whitesides[59] and is based on the idea of combining a biomolecular scaffold (a protein or an oligonucleotide) and a synthetic complex as active site. To date, a variety of biohybrid systems have been reported, offering the possibility to improve the catalytic performances.

The case of hydrogenases is very interesting, in particular for the possibility to optimize electrons and protons transfer. The concept of artificial hydrogenases implies the combination of a well-chosen and easily accessible host protein to a synthetic complex with proton reduction or hydrogen oxidation catalytic activity. The aim is to create a cheap, stable, water-soluble and active hybrid system. The protein would provide an environment of functional groups around the synthetic complex helping proton and electron transfers. For example, Roy et al.[60] managed to synthetize a bioinspired mimic binding a diiron hexacarbonyl cluster to an alanine-rich peptide via an artificial dithiol amino acid. The resulting biohybrid system was able to catalyse photo-induced production of hydrogen in water in acidic conditions and in presence of ruthenium as photosensitizer and ascorbate as sacrificial electron donor. Then, Sano et al.[61] showed that the hybrid system obtained treating apo-cytochrome c with a diiron complex worked as catalyst for hydrogen evolution in presence of ruthenium as photosensitizer and ascorbate as sacrificial reagent in aqueous media.

The fact that in [Fe-Fe]-hydrogenases the diiron cluster is attached to the protein only by a single cysteine allowed considering the direct maturation of an hydrogenase containing all the [4Fe-4S]- clusters but lacking the [2Fe]-subcluster (defined in the following apo-hydrogenase). In particular, HydF has been considered as the most suitable protein for a synthetic bioinspired complex, due to its ability to bind and transfer a diiron cluster. In this regard, it was shown

that incubating HydF from *Thermotoga maritima* with azadithiol (adt-complex), propanedithiol (pdt-complex) and oxapropandithiol (odt-complex) led to hybrid proteins similar to the target metalloenzyme. This result provided the first example of a rational structural design of an artificial hydrogenase. Furthermore, it was observed that only the pdt-hydrogenase showed a small activity in a standard chemical assay, using sodium dithionite and methyl viologen as source of electrons, as well as in a photochemical assay, using a metal–organic photosensitizer and a sacrificial electron donor under visible light irradiation. Although this activity was very low, this is a starting point for the development of more efficient artificial hydrogenases.[58]

Diiron mimics have also been evaluated in non-protein environments, for examples micelles dendrimers, polymers, oligo and polysaccharides and metal-organic frameworks.[62]

Unfortunately, to date these systems are considerably less active than natural enzymes but hope remains that a new, highly active and stable metalloenzyme could be generated, with potential for application in technological devices. This field in fact is still at its infancy and obtaining a working artificial hydrogenase is very complex, as the reactions involve electrons and protons transfers and gas transfer. However, many studies have been and are still being carried out, demonstrating the possibility of creating an active artificial hydrogenase embedding an inefficient catalyst and increasing its efficiency due to the protein environment.

1.8 Aims of the project

1.8.1 HydF from *Thermosipho melanesiensis*

Many studies have been done on HydF structure. The first characterization of HydF was done on the protein from *Thermotoga maritima42,* showing that it contains a [4Fe-4S]-cluster which binds GTP and catalyses its hydrolysis to GDP. Then, FTIR studies provided evidence of the presence of CO and CNligands of the [2Fe] precursor and gel filtration studies of HydEFG showed that the proteins tend to assemble in a dimeric conformation.63 Czech et al. showed that HydF from *Clostridium acetobutylicum* contains an iron cluster with similar structural features as the H-cluster of active HydA from *Chlamydomonas reinhardtii* (CrHydA1).64 The first crystal structure was obtained from HydF from *Thermotoga neapolitana* (TnHydF)*44* and it showed that the protein is organised in three domains: (I) domain I corresponds to the GTP-binding domain, very similar to the other GTP-ases; (II) domain II is connected to domain I by a long stretch of amino acids and it is responsible for HydF dimerization; (III) domain III is the [4Fe-4S]-cluster binding domain and it is connected to this cluster by three highly conserved cysteine residues (Figure 1.8 a). The crystal contained one molecule per asymmetric unit and dimers were obtained by reconstruction of the crystal unit cell. However, the protein was crystallised as a dimer in the sample put to crystallize and a tetramer was formed during the course of the crystallization.

Another crystal structure of HydF was obtained from the [4Fe-4S]-reconstituted protein from *Thermosipho melanesiensis*.[45] The structure contained the three different domains already described for TnHydF (Figure 1.8 b). It was also shown that the iron-sulfur cluster is coordinated

by three very highly conserved cysteines and by the carboxylate group of a glutamate. These residues are located in a positively charged cavity, which fits nicely the need to interact with negatively charged molecules like [4Fe-4S]1-/2- and the [2Fe]-subcluster. Many attempts were done in order to try to obtain crystals with the diiron subcluster, such as co-crystallisation and soaking, but it was not possible to obtain them so far. Interestingly, soaking [4Fe-4S]-TmeHydF crystals in a crystallization cocktail supplemented with the [2Fe] ligands led to the observation of a progressive break of the crystals upon ligand diffusion. Different hypothesis can be made to explain the phenomenon observed. First, it is possible that the diiron complex cannot easily access the cluster because of the presence of the Glu residue. Second, it has been shown that, in the crystal packing of the [4Fe-4S]-TmeHydF, the contact between dimers is in the area where the iron-sulfur cluster and the diiron subcluster are bound, leading to the disruption of the crystals, maybe due to the introduction of a negative charge of the [2Fe]-subcluster. Furthermore, it has been observed that GTP domains have significantly higher B factors with respect to the other two domains (Figure 1.9). As the B factors are related to the mean square isotropic displacement of the atoms, this implies that the GTP domain is not well ordered and is therefore a source of disorder in the crystal, leading to low-resolution crystals.[45]

Figure 1.8 a) Representation of the structure of one subunit of the apo-TnHydF dimer. The three domains, labeled I, II, and III, are in different shades of green and the loop connecting domains I and II is in brown. The side chains of the three cysteines involved in subcluster binding are shown in red.[44] b) Representation of the [4Fe-4S]-HydF dimer from Thermosipho melanesiensis. GTP domains are in green and orange, dimerization domains in magenta and light pink and cluster binding domain in yellow and blue.[45]

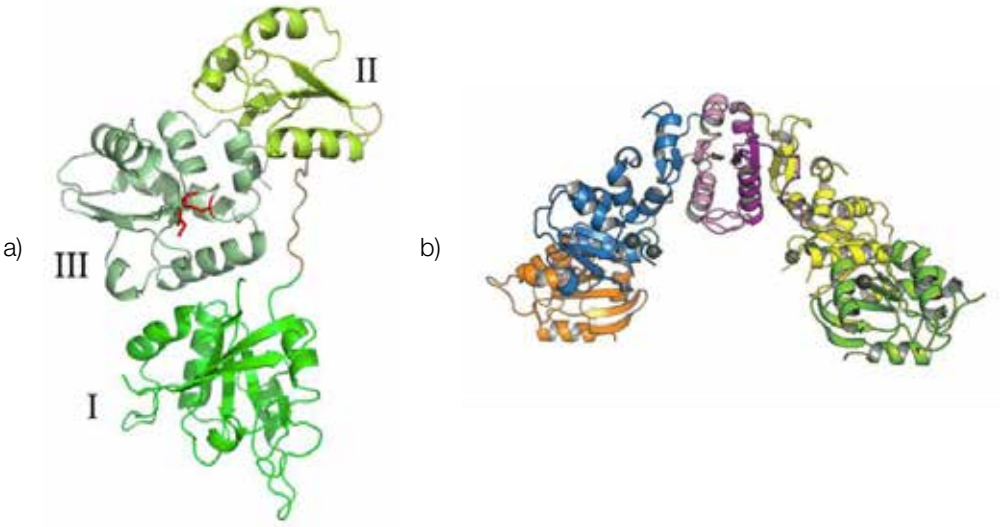
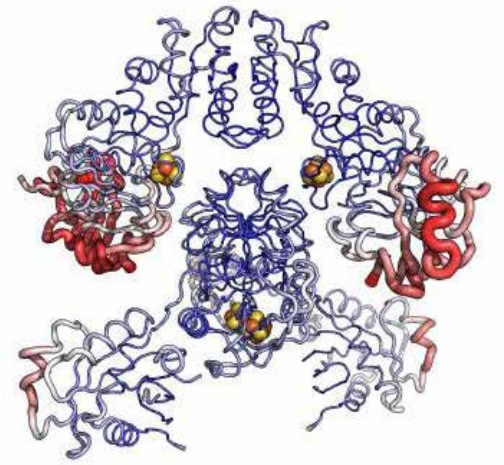


Figure 1.9 B factors of [4Fe-4S]-TmeHydF.



B factors are coloured from blue to red for the range [40:170] Å.[45]

Thus, the first aim of my project was obtaining a crystal structure of HydF containing both the [4Fe-4S]-cluster and the [2Fe]-subcluster. In order to do that, it was necessary to design a truncated form of HydF from *Thermosipho melanesiensis,* cutting the GTP-ase domain from the wild type form. The idea was based on the fact that GTP domains have been observed to be not always good oriented and often disordered areas, causing a low-resolution diffraction of the crystals obtained and being a cause of trouble for the binding of the diiron subcluster.

1.8.2 HydA from *Megasphaera elsdenii*

Structures of HydA from three different microorganisms are already available.

The first structure was obtained from *Clostridium pasteurianum* (CpI) and it was composed of four distinct domains. The largest one is designated as active-site domain and it contains the active-site cluster, known as H-cluster. It is composed of six Fe atoms arranged as a [4Fe-4S]-cluster bridged to a [2Fe]-subcluster by a single cysteine. The [4Fe-4S] cluster is linked to the protein via four cysteines, while the diiron subcluster is composed of two octahedrally liganded Fe atoms that contain five CO/CN ligands, three S ligands and one H2O ligand. The other three domains contain the four accessory iron-sulfur clusters: three [4Fe-4S]-clusters and one [2Fe-2S]-cluster, arranged into three domains.[65]

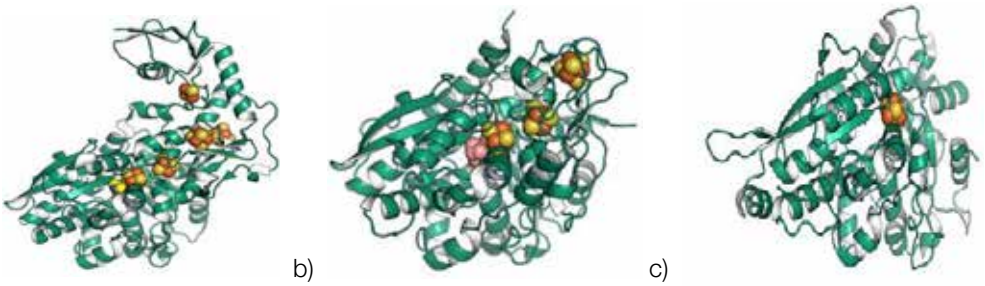
Secondly, it was reported the structure of hydrogenase from *Desulfovibrio desulfuricans* (DdHydA), which presents an identical amino acid sequence with the hydrogenase from *Desulfovibrio vulgaris*. It reveals a conformation for the H-cluster similar to the one solved

for CpI: in fact, it is composed of a ferrodoxin-type [4Fe-4S]-cluster, deeply buried into the protein, and a [2Fe]-subcluster, bridged by a cysteine. Each iron atoms of the binuclear cluster is coordinated to one CO, one CN-, a monoatomic oxygen species and two bridging thiolates. The protein also contains three accessory iron-sulfur clusters, one of which is a [2Fe-2S]-cluster, as found in CpI.[66]

Finally, another structure was obtained from a truncated form of HydA from *Chlamydomonas reinhardtii* (CrHydA1)*,* expressed in absence of the maturases HydE, HydF and HydG and therefore purified in its unmaturated form lacking the [2Fe]-subcluster. The structure presents many similarities with the structures of the other two proteins characterized. CrHydA1 it lacks any accessory cluster and therefore it has been appreciated for its simplicity that can be exploited for investigating maturation of the H-cluster.[67]

The three structures are reported in Figure 1.10.

Figure 1.10 a) Structure of CpI. It is possible to distinguish the first domain containing the H-cluster, composed of a [4Fe-4S] cluster and a [2Fe] subcluster, and the three domains containing the four accessory iron-sulfur clusters. b) Structure of DdHydA. It is possible to distinguish the H-cluster, composed of a [4Fe-4S] cluster and a [2Fe] subcluster, and the three domains containing the two accessory [4Fe-4S] clusters. c) Structure of CrHydA. It contains only one [4Fe-4S] cluster and it lacks the [2Fe] subcluster and the accessory clusters. In each figure, iron atoms are represented in yellow and sulphur atoms are in orange.



Even if these structures are already available and provided important information about the behavior of [Fe-Fe]-hydrogenases, another kind of HydA is being studied. This is the hydrogenase isolated from *Megasphaera elsdenii,* which showed very intriguing and difficulty explaining behaviours in terms of oxygen sensibility. In fact, the laboratory recently showed that it has higher resistance to oxygen, compared to the other HydAs. For this reason, we are interested in studying the structure of this protein in order to understand the cause of its higher oxygen resistance.

So, for the second aim of my project, I focused on the crystallisation of HydA from *M. elsdenii*. In particular, I worked on a truncated form, named MeH HydA, where all the accessory clusters have been eliminated, in order to obtain a simpler protein, in analogy to CrHydA1.

2. Methods and materials

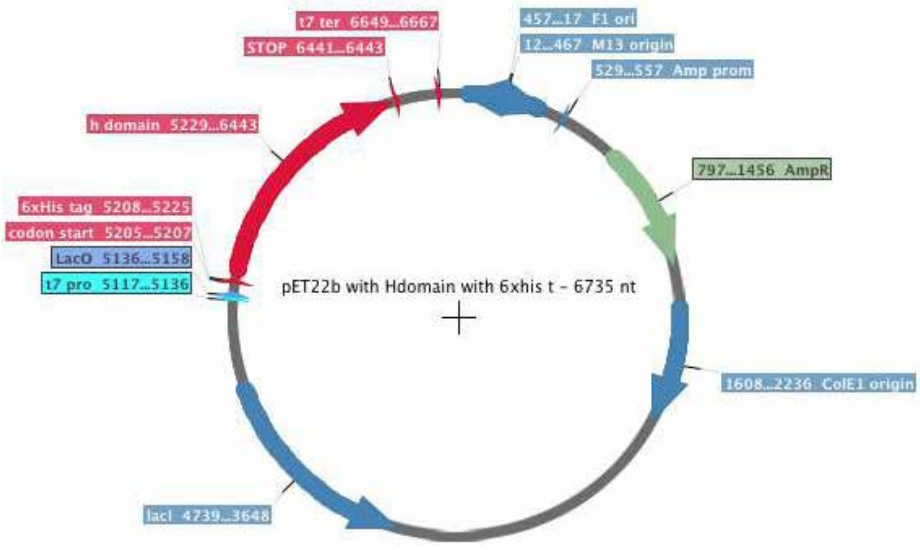
2.1 Heterologous expressions of proteins

A heterologous expression is the expression of a gene in a host organism, which does not produce it naturally. The gene has to be inserted into its expression system through the recombinant DNA technology. The expression system can be a bacterium, a yeast or a plant cell but it has to have a property, known as competence. In molecular biology competence is the skill of a cell to uptake extracellular DNA from its environment. Cells can acquire this property with an artificial process. The laboratory already had competent cells from *E. coli* prepared with chemical method and these cells were used for all the transformations.

2.1.1 Transformation

The transformation is the operation that allows the competent cell to uptake the DNA molecule from the environment, through its plasmatic membrane. The DNA sequence that has to be amplified is contained in plasmids, small circular DNA molecules able to replicate independently. The schematic representation in Figure 2.1 shows an example of plasmid, in particular the pET22b, underlining its main components: it contains a sequence where the replication starts, called origin of replication (in blue), a gene coding for an enzyme giving the antibiotic resistance in order to select the cells that contain the plasmid (ampicillin resistance, in green), the gene of interest (in red) preceded by a t7 promoter (in cyan), necessary for T7 RNA polymerase binding and subsequent protein translation.

Figure 2.1 Schematic representation of pET22b plasmid for MeH HydA with 6 histidine tag.



MeHydA, MeH HydA and ΔTmeHydF without chaperones

The same protocol was used for MeHydA, MeH HydA and ΔTmeHydF without chaperones. Competent cells were transformed with the desired plasmid (respectively TunerDE3pLysS cells transformed with pT7-7-6HMeHydA, TunerBL21 DE3 cells transformed with pET22b and BL21DE3 cells transformed with pET22b plasmid): 1 µL of plasmidic DNA (corresponding to 50-100 ng) was added to 100 µL of cells and left 30 minutes on ice. The cells underwent a heat shock at 42°C for 45 seconds and were incubated 2 minutes on ice. After addition of 400 µL of warm Lysogeny broth (LB) at 37 °C, the cells were incubated at 37°C for 50 minutes. LB was prepared in the laboratory and it is a complex medium containing tryptone as source of peptides, yeast extracts (containing vitamins) and sodium chloride as sodium ions for transport and osmotic balance. Then, the cells were centrifuged at 2000 g for 5 minutes and part of the supernatants was discarded, in order to concentrate the cells. After re-suspension, 20% and 80% of the transformation product were loaded on two agar plates containing Ampicillin 100 µg/mL and incubated overnight at 37°C. In these conditions, only cells containing the plasmid can grow, thanks to the antibiotic resistance provided by beta-lactamase.

ΔTmeHydF with chaperones

For the expression of ΔTmeHydF with chaperones, BL21DE3 cells were used as competent cells. 50 ng of pGRO7plasmid and 50 ng of ΔTmeHydF plasmid were added to 100 µL of cells. Then, the protocol was the same as before. The agar plates contained Ampicillin and Chloramphenicol at 50 and 34 µg/mL respectively.

3. References

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