Activation and sensing of hydrogen in nature

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In the nineteenth century it was already known that certain bacteria in river mud are able to decompose formate into carbon dioxide and hydrogen gas (Hoppe-Seyler, 1887). Bacterial cultures involved in this process were isolated in the early beginning of the twentieth century which led to the recognition of the important role of molecular hydrogen in the metabolism of a range of bacteria. In the 1930’s, Stephenson and Stickland proposed to name the enzyme system responsible for hydrogen metabolism in *Escherichia coli* ‘hydrogenase’ (Stephenson, 1931).

Hydrogenases catalyse one of the simplest reactions in nature, the (reversible) conversion of hydrogen into two protons and two electrons: \( \text{H}_2 \leftrightarrow 2\text{H}^+ + 2e^- \). Enzymes, as biological catalysts, lower the activation energy of a chemical reaction thus accelerating the rate of the reaction, but they do not change the chemical equilibrium. This means that hydrogenases can both consume and produce \( \text{H}_2 \), depending on the conditions. Bacterial cells benefit from the uptake-activity of hydrogenases through the formation of reducing equivalents required for the cell’s metabolism. The soluble hydrogenase from *Ralstonia eutropha*, for example, uses the electrons produced by \( \text{H}_2 \) oxidation for the reduction of NAD\(^+\) to NADH. On the other hand, bacteria can get rid of excess protons via \( \text{H}_2 \)-production catalysed by hydrogenases. For example, the function of the \([\text{NiFe}]\) hydrogenase from *Thiobacillus ferrooxidans*, a bacterium that lives in acidic surroundings, is postulated to be the removal of excess protons from the cytoplasm by the production of \( \text{H}_2 \). This activity helps to maintain a proton gradient over the cytoplasmic membrane and allows the bacterium to survive such harsh conditions.

Although the family of hydrogenases consists of \([\text{NiFe}]\) hydrogenases, \([\text{Fe}]\) hydrogenases and a metal-free hydrogenase, the nickel-containing hydrogenases are the most abundant and the most extensively studied examples of hydrogen-metabolising enzymes. The interest in \([\text{Fe}]\) hydrogenases has strongly increased recently, mainly due to two recent, simultaneous publications of high-resolution crystal structures of the \([\text{Fe}]\) hydrogenases from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* (Peters, 1998; Nicolet, 1999). A recent overview of the \([\text{Fe}]\) hydrogenase literature is presented in (Adams, 2000). The status of the research on the metal-free hydrogenase is reviewed in (Berkessel, 2001). A general overview of the research on all types of hydrogenases can be found in a recent book on this topic (Cammack, 2001).

In the first part of this overview I will address the properties of prototypic \([\text{NiFe}]\) hydrogenases like the ones from *Desulfovibrio gigas* and *Allochromatium vinosum*. These are highly similar enzymes, containing identical cofactors and displaying similar spectroscopic features. An overview of the information gathered in the past decades from spectroscopic and structural studies is presented. In the second part oxygen-tolerant \([\text{NiFe}]\) hydrogenases will be described. These enzymes are able to oxidise \( \text{H}_2 \) under aerobic conditions and contrary to the standard hydrogenases, they are not inhibited by carbon monoxide. A very new class of \([\text{NiFe}]\) hydrogenases are the \( \text{H}_2 \)-sensing hydrogenases described in part three. The main task of these hydrogenases is not to oxidise hydrogen but to sense if \( \text{H}_2 \) is present, in which case the whole machinery that is required for the proper biosynthesis of the \( \text{H}_2 \)-oxidising hydrogenases, is switched on. Finally, the complex protein machinery that is required for correct active-site assembly is described in part four. After assembly and processing in the cytosol, the periplasmic \([\text{NiFe}]\) hydrogenases utilise a novel type of translocation machinery to cross the membrane: the so-called TAT (twin-arginine translocation) pathway. This system is also briefly described in the last part of this introduction.
1.1 Standard [NiFe] hydrogenases

A variety of [NiFe] hydrogenases belongs to the group of standard hydrogenases (e.g. ferredoxin:H⁺ oxidoreductase, E.C. 1.18.99.1; cytochrome-c hydrogenase, E.C. 1.12.2.1), although nowhere the exact prerequisites for such a qualification are defined. Mostly, the definition is used in the way that if the enzyme described has similar properties as the rest of the well-studied hydrogenases it is grouped in the class of standard hydrogenases. In practice this means that any new member is compared to the most extensively studied enzymes like the [NiFe] hydrogenases from *D. gigas* and *A. vinosum*. From the biochemical and spectroscopic point of view, the [NiFe] hydrogenases from these sources are very similar, as will be described in this part.

1.1.1 Biochemical properties

The prototypic [NiFe] hydrogenases share the basic biochemical characteristics. They consist of two subunits, a large one of approximately 60 kDa and a small one of about 30 kDa and contain 12 Fe atoms per 1 Ni atom. The large subunit harbours the active site, which consists of two metal ions, a Ni and an Fe ion. The small subunit contains three Fe-S clusters, two [4Fe-4S] clusters and one [3Fe-4S] cluster. A large number of amino acid sequences of [NiFe] hydrogenases have been determined from the structural genes encoding the two subunits. Analysis of their sequence similarities revealed that the [NiFe] site-binding, large subunits are fairly conserved but that the sequences of the Fe-S cluster-binding, small subunits show comparatively more variation (Albracht, 1994). In some cases one of the cysteins that coordinates the active site is replaced by a selenocystein. The resulting hydrogenase is named a [NiFeSe] hydrogenase.

H₂-oxidation occurs heterolytically which means that H₂ is split into a hydron (H⁺) and a hydride (H⁻) (Krasna, 1954). The specific activity of active [NiFe] hydrogenases from *D. gigas* with a redox dye like methyl viologen is 300 Units/mg at pH 8.5 and at 30°C (Fernandez, 1985). This value is generally found for the H₂-uptake activity of standard [NiFe] hydrogenases using a redox dye as electron acceptor, and corresponds to a turnover number of about 450 s⁻¹. However, provided [NiFe] hydrogenases can dispose of produced electrons quick enough, their H₂-oxidising activity is limited by H₂ diffusion. For the *A. vinosum* MBH immobilised at the surface of a fast-rotating graphite electrode, turnover numbers of 10⁷ - 10⁸ s⁻¹ have been measured (Pershad, 1999).

A general property of standard [NiFe] hydrogenases is that they are reversibly inactivated by oxygen, which allows aerobic purification. After aerobic purification the enzyme is inactive and (at least partially) EPR detectable. EPR detectable aerobic enzyme can be in two different states, the 'unready' or the 'ready' state. The nomenclature of these states is based on its behaviour in an activity assay: enzyme in the 'ready' (Niᵣ) state displays its activity after a short lagphase of 1 to 2 minutes whereas enzyme in the 'unready' state cannot activate under these conditions (Fernandez, 1985). Oxidised enzyme can be activated by prolonged incubation under hydrogen, preferably at elevated temperature, a process known as 'reductive activation'. Activation of *A. vinosum* [NiFe] hydrogenase could be prevented by cooling of the enzyme sample to 2°C (Coremans, 1992b). Obviously, H₂ oxidation by these enzymes requires
anaerobic conditions. Reduced, active [NiFe] hydrogenases are inhibited by carbon monoxide \( (K_r \sim 30 \, \mu \text{M}) \) \cite{Berlier1987).

1.1.2 Structural properties

**Active site.** Currently, six different [NiFe] hydrogenases from several sources and in various redox states have been crystallised, yielding a wealth of structural information. The first structure that was solved and published in 1995 was the [NiFe] hydrogenase from \textit{D. gigas} in an oxidised form. The resolution of this structure is 2.85 Å and it shows the active site bound to the large subunit and deeply buried inside the protein. The active site is located very close to the contact surface of the large and the small subunit and, surprisingly at the time, it contains two metal ions (Figure 1). One of them was identified as a nickel ion, whose presence was already forecasted by spectroscopic studies and the second metal site was putatively assigned to contain iron \cite{Volbeda1995}. The bimetallic centre is attached to the protein via four cystein residues: two coordinate end-on to the Ni (Cys65 and Cys530) and two thiols bridge the Ni and the Fe (Cys533 and Cys68). A third bridging ligand is proposed to be an oxygen species (Figure 2). The Fe ion binds three diatomic non-protein ligands, initially modelled as water ligands (L1, L2 & L3 in Figure 1, \textit{Volbeda}, 1995). In a succeeding study the structure was refined to 2.54 Å and the second metal was positively identified to be Fe by an X-ray study on the Fe absorption edge \cite{Volbeda1996a}.

In an FTIR study on the \textit{A. vinosum} enzyme in the CO-inhibited state, three absorptions were discovered in the 2100-1900 cm\(^{-1}\) region \cite{Bagley1994}. An FTIR study on [NiFe] hydrogenase purified from \textit{A. vinosum} cultivated on media enriched with \(^{13}\text{C}\) and/or \(^{15}\text{N}\) led to the identification of the three non-protein ligands to Fe as one carbon monoxide and two cyanides (Figure 2, \textit{Happe}, 1997; \textit{Pierik}, 1999). The presence of these ligands also explained the low-spin ferrous iron observed in the \textit{A. vinosum} enzyme by Mössbauer spectroscopy \cite{Surerus1994}.

![Figure 1. Array of the prosthetic groups in the [NiFe] hydrogenase from \textit{D. gigas}. The diatomic molecules coordinated to the Fe site were modelled as water ligands (L1, L2 & L3). Reprinted with permission from \textit{Volbeda, Curr. Opin. Struct. Biol.} 6, 804-812. Copyright 1996, Elsevier Science.](image)

In addition to the crystal structure of the \textit{D. gigas} [NiFe] hydrogenase, the structures of similar hydrogenases from different bacterial sources have been solved. All these structures but one show similar features. The odd one out is the [NiFe] hydrogenase from \textit{Desulfovibrio vulgaris} strain Miyazaki, the X-ray structure of which has been solved at a resolution of 1.8 Å \cite{Higuchi1997}. The general protein structure is similar to that of the \textit{D. gigas} enzyme, but there are two clear differences in
interpretation. Firstly, the bridging ligand, proposed to be an oxygen species in the \textit{D. gigas} hydrogenase, was modelled as a sulphur ligand in the enzyme from \textit{D. vulgaris} (Higuchi, 1997). This assignment is supported by the observation that upon reduction with hydrogen the bridging ligand is removed (Higuchi 1999a) and H$_2$S is liberated from the enzyme (Higuchi, 1999b). Secondly, the three diatomic non-protein ligands to the Fe site are modelled as SO, CO and CN' (Higuchi, 1997, 2000a). The characterisation of one of the diatomic ligands as a SO is rather controversial. The infrared spectrum from this enzyme (Higuchi, 2000a) is very similar to those published for \textit{A. vinosum} and \textit{D. gigas} [NiFe] hydrogenases (Bagley, 1994; Volbeda, 1996a). For the \textit{A. vinosum} [NiFe] hydrogenase it has unequivocally been demonstrated that the two absorption peaks in the cyanide region are caused by the coupled vibrations of two cyanide ligands (Pierik, 1999). Nevertheless, in mass spectrometry experiments on the \textit{D. vulgaris} [NiFe] hydrogenase a peak attributable to SO has been observed (Higuchi, 2000a) but this might originate from an oxidative degradation of, for example, cysteine residues in the enzyme.

\begin{center}
\includegraphics[width=0.5\textwidth]{structure.png}
\captionof{figure}{Structure of the active site of standard [NiFe] hydrogenases. Adapted from Happe, 1997.}
\end{center}

\textbf{Electron- and proton-transfer pathways.} Since the active site is buried in the centre of the protein, electrons and protons must travel a distance of about 30 Å between the active site and the surface of the protein. To facilitate transport of these charged particles, [NiFe] hydrogenases contain specific transport routes. The small subunit of the \textit{D. gigas} [NiFe] hydrogenase contains three Fe-S clusters: two [4Fe-4S] clusters and one [3Fe-4S] cluster. They are in an almost linearly alignment from the active site to the surface of the protein with an average cluster-to-cluster distance of 12 Å (Volbeda, 1995). The proposed function of the Fe-S clusters is to transfer electrons from the active site to an electron acceptor on the surface of the protein. The [4Fe-4S] cluster closest to the active site (~10 Å from Ni) is named the proximal cluster. The [3Fe-4S] cluster is located between the proximal and the distal cluster, which is the one closest to the surface of the molecule (Figure 1). Along with electrons, also protons are produced in the oxidation of hydrogen. These protons also have to be transported to the outside of the enzyme and a possible proton transport chain has been proposed in the crystal structure of the \textit{D. gigas} [NiFe] hydrogenase (Figure 1). Protons move inside proteins through displacements of ca. 1 Å that are accommodated by small rotations and vibrations of proton donor and acceptor groups like histidines, carboxylates and internal, structural water molecules. A possible proton transport pathway has been recognised in the crystal structure from \textit{D. gigas} hydrogenase but has not yet been confirmed by experimental evidence.

\textbf{Hydrogen-gas accessibility to the active site.} To allow hydrogen gas to enter the active site, the protein contains hydrophobic gas channels. The accessibility of these cavities was calculated for a probe radius
of $1 \, \text{Å}$, the size of an $\text{H}_2$ molecule. Three orifices on the enzyme surface allow gases to enter and inside the protein these three channels join to end near the Cys530 residue, close to the open binding place on the Ni ion. Experimentally, the channels were visualised in the $[\text{NiFe}]$ hydrogenase of Desulfuricans fructosovorans by exposing the protein crystals to Xenon gas under high pressure (9 bar). Xe has the ability to interact with protein and due to its high mass it can be detected in difference Fourier electron density maps (Montet, 1998). This study shows ten Xe binding-sites in the calculated hydrophobic cavities in the protein. Simulation of $\text{H}_2$ diffusion through the protein to the active site using molecular dynamics shows that the $\text{H}_2$ molecules never diffuse randomly through the protein but instead always diffuse through the calculated hydrophobic channels (Montet, 1998).

**Crystal structures of reduced enzyme.** A $[\text{NiFeSe}]$ hydrogenase from Desulfomicrobium baculatum, has been crystallised in the reduced state, in the presence of $\text{H}_2$ (Garcin, 1999). The overall protein structure is very similar to the structures published before, but there is one major difference: the bridging ligand, present in all structures of oxidised $[\text{NiFe}]$ hydrogenases, is absent in the structure of the reduced enzyme. A similar result was found for the $D. \text{vulgaris}$ Miyazaki F $[\text{NiFe}]$ hydrogenase (Higuchi, 1999a) and the $D. \text{gigas}$ [NiFe] hydrogenase (Fontecilla-Camps, 2001) in the reduced states. This shows that one of the steps involved in the activation process is the removal of the bridging ligand. The $D. \text{vulgaris}$ Miyazaki F hydrogenase has also been crystallised in the reduced, CO-inhibited state (Higuchi, 2000b). The presented structure confirmed the assumption that the Ni ion is the binding site for CO. The CO molecule binds in an end-on way to the Ni ion, pointing towards the gas channel. It thus blocks binding of $\text{H}_2$ molecules to the active site and prevents enzyme activity.

### 1.1.3 Spectroscopic properties

Due to the presence of various transition metals in $[\text{NiFe}]$ hydrogenases, a variety of spectroscopic techniques has been used to study both the structural and the redox properties of the metal-sites in the protein. Applied techniques vary from well-established techniques like EPR to novel applications of soft X-rays like L-edge spectroscopy. In this part a short overview is given on the development of the spectroscopic studies on $[\text{NiFe}]$ hydrogenases.

**Electron paramagnetic resonance (EPR) spectroscopy.** Initially, hydrogenases were only recognised to contain Fe arranged in Fe-S clusters and EPR was mostly used to study the paramagnetic states of the Fe-S clusters. In addition to EPR, also techniques like Mössbauer and UV-Vis spectroscopy were applied to investigate the characteristics of the Fe-S clusters. Mössbauer spectroscopy on oxidised $[\text{NiFe}]$ hydrogenase from $D. \text{gigas}$ enriched in $^{57}\text{Fe} \, (I = 1/2)$ showed two diamagnetic ($S = 0$) $[4\text{Fe}-4\text{S}]^{2+}$ clusters (Teixeira, 1989). In addition, also a paramagnetic $[3\text{Fe}-4\text{S}]^{1+}$ cluster was observed in the oxidised state (Teixeira, 1989). The rather isotropic EPR spectrum associated to this paramagnetic cluster ($S = 1/2$) was centred round $g = 2$ and showed a large amplitude.
After incubation under H₂, this intense signal disappeared and very broad signals due to reduced [4Fe-4S]⁺ clusters (S = 1/2) were observed (Cammack, 1982). The reduced [3Fe-4S] cluster is in an S = 2 spin-state, showing a low-field feature at g = 12 (Teixeira, 1989). The midpoint potentials of the two [4Fe-4S] clusters were found to be fairly low (-400 and -350 mV at pH 8) in the D. gigas hydrogenase (Teixeira, 1989). That of the [3Fe-4S] cluster, however, was a lot higher at -35 mV (pH 7) in the D. gigas enzyme (Cammack, 1982).

In the first low-temperature EPR studies, the signal due to a nickel-based unpaired electron was overlooked (Strekas, 1980). Since the rhombic signal only showed a relatively small amplitude, the Ni signal was overshadowed by the fairly intense signal of the oxidised [3Fe-4S] cluster. Only in 1982 the unequivocal proof for the presence of Ni in the enzyme was presented (Albracht, 1982). The EPR spectrum of the [NiFe] hydrogenase of Methanobacterium thermoautotrophicum strain Marburg (nowadays named Methanothermobacter marburgensis) enriched in ⁶⁷S (I = 3/2) showed a clear broadening of the g₁ line (at 2.31) and split lines at 2.24 (g₂) and 2.01 (g₃). After incubation of the enzyme with H₂, this signal disappeared, for the first time indicating a redox active Ni site in this enzyme. An EPR study on the [NiFe] hydrogenase of Wollinella succinogenes enriched in ³²S (I = 3/2) showed that enrichment in this nucleus clearly broadened both the spectrum of the oxidised [3Fe-4S] cluster and the spectra in the oxidised and reduced EPR detectable states (Albracht, 1986). From this hyperfine interaction it was concluded that the Ni-site was coordinated by at least one, probably more, sulphur ligands in the active site and that the unpaired electron from Ni was partly localised on the neighbouring sulphur atoms (Albracht, 1986).
As described already the Ni site is redox active and, when oxidised, can occur in different states: the Ni\textsuperscript{'\textsubscript{r}} and the Ni\textsuperscript{\textsubscript{u}} state, both showing EPR spectra associated with a Ni\textsuperscript{2+} (3d\textsuperscript{'} ion. Their EPR spectra are rather similar and mainly differ in their g value: 2.16 in the Ni\textsuperscript{1\textsuperscript{'}\textsubscript{r}} and 2.24 in the Ni\textsuperscript{\textsubscript{u}} state (see Figure 3). Oxidation of active A. vinosum MBH with O\textsubscript{2} enriched in \textsuperscript{17}O (nuclear spin, I = 5/2) caused a broadening of the EPR signals of both the Ni\textsuperscript{1\textsuperscript{'}\textsubscript{r}} and the Ni\textsuperscript{\textsubscript{u}} state (Van der Zwaan, 1990). It was concluded that in both states an oxygen species, located near the Ni-based unpaired electron is blocking the active site, preventing hydrogen oxidation. It is now generally accepted in the field that this ligand is the third bridging ligand observed in the crystal structures of oxidised enzyme. Recent ENDOR measurements on the [NiFe] hydrogenase from D. gigas oxidised in H\textsubscript{2}\textsuperscript{17}O showed that this oxygen ligand can also originate from bulk water (Carepo, 2001). EPR spectroscopy on single crystals yields information about the orientations of the g-tensors. It was shown that the g-tensors were similarly oriented in both the Ni\textsuperscript{1\textsuperscript{'}\textsubscript{r}} and the Ni\textsuperscript{\textsubscript{u}} state (Geßner, 1996; Trofanchuk, 2000).

After reduction with H\textsubscript{2}, standard [NiFe] hydrogenases display a third EPR detectable state, called the Ni\textsuperscript{\textsubscript{u}}-C\textsuperscript{'} state (Van der Zwaan, 1985). The EPR spectrum of this state is slightly less rhombic (Figure 3, g\textsubscript{\textsuperscript{\textsubscript{r}} = 2.21, 2.16, 2.01) than those of the oxidised states and the Ni ion is thought to be 3+ in this state (Moura, 1982; Happe, 1999). An interesting feature of enzyme in the Ni\textsuperscript{\textsubscript{u}}-C\textsuperscript{'} state is its light-sensitivity at low temperatures (Van der Zwaan, 1985). Upon illumination at low temperatures (<77 K) the spectrum changes, its g values shifting to 2.30/2.28, 2.11 and 2.045 (Figure 3), and the Ni\textsuperscript{\textsubscript{u}}-C\textsuperscript{'} state is converted to the Ni\textsuperscript{1\textsuperscript{'}\textsubscript{u}}-L\textsuperscript{'} state, a light-induced state. When the Ni\textsuperscript{\textsubscript{u}}-C\textsuperscript{'} state is prepared in D\textsubscript{2}O, the effect of the illumination is six-fold slower. This isotope effect is indicative of the involvement of a photolabile hydrogen species in this process. It is postulated that the conversion of Ni\textsuperscript{\textsubscript{u}}-C\textsuperscript{'} to Ni\textsuperscript{1\textsuperscript{'}\textsubscript{u}}-L\textsuperscript{'} involves a reduction of the Ni ion from Ni\textsuperscript{3+} to Ni\textsuperscript{2+} (Happe, 1999; Stein, 2001a), the required electrons being supplied by the photolysed hydride. The reduced states also show a split spectrum at low temperatures due to the interaction with the reduced proximal Fe-S cluster (Figure 3).

**Fourier-transform infrared (FTIR) spectroscopy.** As mentioned before, carbon monoxide is a potent inhibitor of standard [NiFe] hydrogenases. In the case of CO-inhibited [NiFe] hydrogenases the Ni site is divalent and thus cannot be monitored by EPR (Van der Zwaan, 1986). In the early nineties, the Amsterdam group, in collaboration with Woodruff and co-workers in Los Alamos, decided to use FTIR as a method to monitor the CO-inhibition of the [NiFe] hydrogenase from A. vinosum. Again serendipity proved to be an important factor in science when, instead of the expected single peak due to the stretch vibration of the inhibiting CO, three extra peaks were detected in the region of 2100-1900 cm\textsuperscript{-1} (Bagley, 1994). The use of \textsuperscript{13}CO allowed the assignment of one of the smaller peaks (at 2060 cm\textsuperscript{-1}) to the inhibiting CO (Bagley, 1994). The inhibiting CO molecule could be photolysed with white light at low temperatures (20 K), but apart from the removal of the absorption peak at 2060 cm\textsuperscript{-1} this hardly affected the remaining three absorptions (Bagley, 1994). After warming the sample to 200 K for more than 10 min the original spectrum was regained, illustrating the reversibility of the photolysis. It was also shown that oxidised [NiFe] hydrogenase, that had not been treated with CO, already showed three absorption peaks, two smaller ones and a large one (Bagley, 1994). In a subsequent study it was shown that the three intrinsic absorption bands were sensitive to changes in the redox state of the enzyme (Bagley, 1995). In Figure 4 an up-to-date overview is presented of the absorption bands and
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how they correlate to various redox states. Speculations about the nature of the peaks at the time included groups containing either triple bonds or adjacent double bonds, such as nitriles, cyanide or azide groups (Bagley, 1995). Around the same time the first crystal structure was published (Volbeda, 1995) with its a binuclear active site and three non-protein ligands coordinated to the Fe site. These ligands, modelled as water molecules, were considered potential candidates for the three IR detectable groups (Bagley, 1995). A survey study of a large amount of metal-containing enzymes (e.g. CO-dehydrogenase, Complex I, ferredoxins, Rieske proteins) showed that the presence of these IR detectable groups was restricted to [NiFe] and [Fe]-only hydrogenases from various sources (Van der Spek, 1996). At the same time it was also shown that the D. gigas [NiFe] hydrogenase used in the crystallisation experiments, contained similar absorptions in this region of the IR (Volbeda, 1996a). The redox properties of the D. gigas hydrogenase were also studied using an electrochemical cell, clearly illustrating the dependency of the peak positions on the redox potential (De Lacey, 1997). A schematic overview of FTIR spectra associated with various redox states of the A. vinosum [NiFe] hydrogenase is presented in Figure 4.

![Schematic overview of FTIR spectra](image)

Figure 4. Overview of the CN' and CO stretching frequencies observed for the various states of the A. vinosum [NiFe] hydrogenase. The positions of the symmetric and anti-symmetric stretching frequencies of the coupled CN vibrations and of the stretching frequency of the CO vibrations are shown. The band due to exogenously bound CO is depicted in grey. A similar but less detailed figure was published in Bagley, 1995.

In order to establish the identity of the non-protein ligands, the [NiFe] hydrogenase from A. vinosum was purified from a batch grown on a medium enriched in $^{13}$C: all three peaks shifted to lower wavenumber indicating that carbon was a constituent of all three ligands. In the FTIR spectrum of A. vinosum [NiFe] hydrogenase purified from bacteria grown on a medium enriched in $^{15}$N only the two smaller peaks displayed a shift to lower wavenumber (Happe, 1997). This showed that the low-intensity absorption bands were due to a diatomic ligand containing C and N. The observed shifts
were reproduced using Hooke's law for an approximate harmonic oscillator for a CN⁻ ligand. The mass of the counter atom of the group vibrating at 1944 cm⁻¹ could also be calculated from the observed spectral shift. The high-intensity absorption band at 1944 cm⁻¹ turned out to be due to a CO ligand. Taken together this resulted in a model in which two cyanides and one carbon monoxide caused the observed absorption in the IR (Figure 2, Happe, 1997). A combination of the crystallographic and the FTIR data lead to the proposal of the active site structure as depicted in Figure 2. In a later study a quantitative chemical analysis showed that the A. vinosum [NiFe] hydrogenase contained 1 CO and 2 CN⁻ ligands per Ni atom (Pierik, 1999). Moreover, by enriching the enzyme for only 50% with ¹⁵N, it was elegantly shown that the vibrations of the two cyanides are coupled, resulting in a symmetric and an antisymmetric stretch vibration observed as two absorption peaks in the IR (Pierik, 1999).

Combining the results of (mainly) EPR and FTIR spectroscopy yields the schematic overview of the identified redox states. The most oxidised states are depicted at the top, the most reduced states at the bottom. The inactive states are separated from the active states by an energy barrier. Information about the number of electrons and protons involved in the various transitions was obtained from redox titrations.

Figure 5. Overview of the identified redox states of the active site in the [NiFe] hydrogenase from A. vinosum as derived from redox titration experiments in the presence of redox mediators. The observed transitions involve both electrons (e⁻) and protons (H⁺) as indicated on the left of the figure. Reduced states can also rapidly interact with H₂ as indicated on the right of the figure. Dotted arrows indicate a slow reaction. Similar states were observed in D. gigas [NiFe] hydrogenase (DeLacey, 1997). An asterisk indicates an EPR-detectable state, S indicates an EPR-silent state.

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**X-ray absorption spectroscopy (XAS).** The Ni centres of a number of [NiFe] hydrogenases from various sources have been studied with X-ray absorption spectroscopy. High-energy synchrotron radiation (~8,300 eV) is used to photo-emit an electron from the K-edge from a Ni atom (or ion) to the continuum. An advantage of this technique over, for example, EPR is that the ion of interest can always be detected, irrespective of its valence state. To obtain informative data, however, rather homogeneous redox states (>80%) are required. Another problem with XAS is that, if no structural data are available, it is difficult to account for changes observed in edge- or fine-structure. Structural information can be derived from pre-edge features and from the fine structure of the XAS spectrum. X-ray absorption near edge structure (XANES) arises from spin-forbidden electron transitions like 1s → 3d transitions. The intensity of this sort of transitions depends on the local symmetry around the metal ion and therefore carries information about the structure. The extended X-ray absorption fine structure (EXAFS) of the absorption edge is determined by the interference of the outgoing photoelectron with the back-scattered photoelectrons by ligands around the metal. Thus, information about the number and nature of the ligands and their respective distances to the metal-ion can be derived from this region of the XAS spectrum.

![Image of X-ray absorption spectra](image)

Figure 6. Left panel: overview of the Ni K-edge X-ray absorption spectra of the [NiFe] hydrogenase from A. vinosum poised in various redox states: Ni\(^{2+}\) (Form A), Ni\(^{2+}\) (Form B), Ni\(^{2+}\)-S (SI\(_A\)), Ni\(^{2+}\)-S (SI\(_B\)), Ni\(^{2+}\)-C\(^{2-}\) (Form C), Ni\(^{2+}\)-L\(^{2-}\) (Form L), Ni\(^{2+}\)-SR (R) and Ni\(^{2+}\)-S\(^{2-}\)CO (SI-CO). Right panel: Fourier-transformed EXAFS regions of the spectra in the left panel.

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Examination of Ni K-edge spectra from [NiFe] hydrogenase revealed only a small shift (~1 eV) between the most oxidised and the fully reduced state (Bagyinka, 1993; Gu, 1996). In Figure 6, XAS data are presented for the A. vinosum [NiFe] hydrogenases poised in various redox states (Davidson, 2000). Also the EXAFS data for this enzyme in various states are shown in Figure 6. This elaborate study, a collaboration of our laboratory with the group of Maroney, showed similar K-edge
absorption spectra for most of the redox states, indicating that the structure of the Ni site only slightly changes (Davidson, 2000). In model compounds a change in valence state of Ni is accompanied by a shift of the Ni K-edge of ~2 eV (Kirby, 1981; Maroney, 1991). The largest change in Ni K-edge, however, was between the Ni$^+$ state and the one-electron reduced Ni$_2$-S state and was only 1.6 eV. This transition was also accompanied by a large change in the EXAFS region (Figure 6). Since this change could be modelled as the loss of an oxygen ligand at short distance from Ni (1.9 Å) the Ni$^+$ to Ni$_2$-S transition was proposed to be the step in which the bridging ligand was removed and the enzyme activated (Davidson, 2000).

A rather new technique to study transition metals in proteins is L-edge X-ray absorption spectroscopy (Wang, 2000). In this technique soft X-rays (~850 eV) are used to excited electrons from a 2p orbital to the 3d orbital. Since this transition is symmetry allowed, the observed absorptions are much more intense. Therefore, one of the advantages of this technique is that less sample is required for L-edge XAS (20 µl, 200 µM) than for K-edge XAS (500 µl, 0.5–1.0 mM). L-edge XAS yields information about both the valence state of the metal ion under study and its spin-state. The most extensively studied [NiFe] hydrogenase is thus far the one from $D$. gigas (Figure 7). It was poised in different redox states and, among other results, it was confirmed that the Ni ion in oxidised enzyme is in a low-spin 3+ state (Wang, 2000). A more interesting observation, however, was that in the CO-binding Ni$_2$-S-CO state the divalent Ni ion was apparently in a high-spin state (Wang, 2000). The amount of evidence supporting the occurrence of high-spin Ni(II) centres in [NiFe] hydrogenase is increasing (Fan, 2002; DeLacey, 2002). To increase the applicability of this technique, development of more sensitive detectors and standardised measurement methods are being developed (Wang, 2001).

![Figure 7. Examples of L-edge spectra from [NiFe] hydrogenases: (a) as-isolated enzyme from $D$. gigas (solid line) compared with H$_2$-reduced $D$. gigas enzyme (dashed line); (b) as-isolated (dashed) and H$_2$-reduced (solid) enzyme from Desulfovibrio baculatus and (c) oxidised (dashed) and H$_2$-reduced (solid) enzyme from Pyrococcus furiosus. Reprinted with permission from Wang, J. Am. Chem. Soc. 122, 10544–10552. Copyright 2000, American Chemical Society.](image)
1.1.4 Theoretical studies

With the structural data in hand several groups started to calculate the lowest energy conformations for the binuclear active site. The first study was published in 1998. In this study the protein surrounding the active site was completely ignored and the active site atoms were allowed to move freely through a gas phase to optimise the geometry to the lowest energy transition state required for the oxidation of hydrogen (Pavlov, 1998). This initial study resulted in a highly unlikely mobility of one of the cyanide groups and hence clearly illustrated the necessity of the incorporation of some structural constraints on the active site, as normally applied by the protein. In a later study this fact was acknowledged and the ligands were fixed in space (Pavlov, 1999), in agreement with the geometry observed in the crystal structure of reduced hydrogenase (Garcin, 1999).

The significance of this latter premise was shown in a recent study (Fan, 2002). In this study the spin-state of the Ni ion in two of the EPR silent states (Ni₆-S and Ni₆-SR) was investigated. The active site was modelled as Ni⁺⁺ (a 3d⁴ ion) in the low-spin (LS, S = 0) and in the high-spin state (HS, S = 1). This showed that, if the positions of the atoms were restricted to the coordinates observed in the crystal structure of enzyme in the reduced state, the complex lowest in energy, rather surprisingly, corresponded to a HS state. The geometry of divalent four-coordinated Ni tends to be square planar and this is also the geometry to which most of the proposed models for the silent states converge (see also Fan, 2001).

Calculations based on density function theory (DFT) nowadays seem to be the general approach in the theoretical studies (Niu, 1999, 2001; Li, 2001). These involve geometry optimisations using the Becke exchange and Lee-Yang-Parr correlation functionals (BLYP) in conjunction with a special basis set for all atoms in the complex. The modelled complex usually consists of the active site metals, the binuclear ligands and methylated sulphurs mimicking the four cysteins that coordinate the active site to the protein. The total complex consists of approximately 30 atoms. From these DFT calculations, structural models have been predicted for numerous redox states of standard [NiFe] hydrogenases (Figure 8).

Stein et al. showed that the inclusion of relativistic effects in the calculations can likewise result in reasonable estimations of g tensors and attendant valence states (3d⁴ or 3d⁵ ions) in the paramagnetic states (Stein, 2001a). In another study on [NiFe] hydrogenases in the Ni₆-C⁻ state they showed that relativistic DFT calculations can also yield estimates for atomic spin populations and hyperfine coupling constants (Stein, 2001c). This is very useful since via this information also a non-specialist can get some insight into the accuracy of the description of the active site by comparing the calculated parameters to the available spectroscopic data. The general message of the foregoing is that a theoretical approach to the understanding of the catalytic mechanism of [NiFe] hydrogenases is certainly a useful one, but that great care must be taken that the proposed, calculated structures are in agreement with the experimental data.
Figure 8. Overview of the structures of [NiFe] hydrogenases proposed on the basis of DFT calculations. The nomenclature used in this figure is different from the nomenclature used throughout this thesis. Structures for the following states are presented: Ni' (Ni-A), Ni-S (Ni-SU), Ni' (Ni-B), Ni-S (Ni-SR), Ni-S (Ni-SIa), Ni-C (Ni-C), Ni-SR (Ni-R), Ni-L' (Ni-L) and Ni-S-CO (Ni-CO). The data were derived from Niu, 1999 and Li, 2001 (I) and from Stein, 2001a, 2001b, 2001c and 2001d (II). Reprinted with permission from Stein, Curr. Opin. Chem. Biol. 6, 243-249. Copyright 2002, Elsevier Science.
1.2 Oxygen-tolerant [NiFe] hydrogenases

The standard [NiFe] hydrogenases described before are only capable of catalysing the reversible heterolytic dissociation of molecular hydrogen under anaerobic conditions. Some [NiFe] hydrogenases, however, are able to catalyse this reaction in the presence of oxygen. Not only are they not affected by oxygen, also CO, a common inhibitor of standard [NiFe] hydrogenases, does not seem to lower the activity of these enzymes. The soluble [NiFe] hydrogenase (SH) from the chemolithotrophic bacterium *R. eutropha* is the best studied oxygen-tolerant hydrogenase and will be the paradigm for oxygen tolerant hydrogenases in the following part. *R. eutropha* contains a second H₂-oxidising enzyme, a membrane-associated periplasmic hydrogenase, the MBH. This enzyme is linked to the membrane via a *b*-type cytochrome that accepts the produced electrons and transfers them to the electron transfer chain in the membrane. Since the MBH also functions under aerobic conditions it is most probably, like the SH, able to oxidise H₂ in the presence of O₂.

Tolerance toward oxygen is a prerequisite for the hydrogenases from *R. eutropha* since this bacterium grows under aerobic conditions. *R. eutropha* can grow chemolithotrophically, using CO as carbon source and H₂ as energy source (Schlegel, 1976). It is a so-called ‘Knallgas’ bacterium since it can reduce oxygen directly to water with the use of hydrogen: 2H₂ + O₂ → 2H₂O. Evidently, a hydrogenase that can function under these conditions must be able to withstand the presence of oxygen. As a matter of fact, the manner in which the active site of the [NiFe] hydrogenase from *R. eutropha* is protected from oxygen is one of the topics addressed in this thesis.

1.2.1 Biochemical properties

The SH (hydrogen:NAD⁺ oxidoreductase, E.C. 1.12.1.2) from *R. eutropha* is located in the cytoplasm of the bacterial cell. It is a heterotetramer consisting of two separate domains: a hydrogenase and a diaphorase domain, both dimers. The hydrogenase domain consists of a large subunit (54.8 kDa) harbouring the binuclear active site, and a small subunit (22.9 kDa) containing a [4Fe-4S] cluster and a domain to which an FMN moiety is rather loosely bound (Van der Linden, 2002). The second part of the complex is the diaphorase domain also consisting of 2 subunits, a small one (26.0 kDa) with one [4Fe-4S]¹⁺⁺⁺⁺ cluster and a large one (66.8 kDa) containing a [2Fe-2S]⁺⁺⁺⁺ cluster, a [4Fe-4S]⁰⁺⁺⁺⁺ cluster and the second FMN (Schneider, 1978; Erkens, 1996). Produced electrons are transferred to the FMN site via the Fe-S clusters where they are used to reduce NAD⁺ to NADH, providing reducing equivalents to the organism.

Aerobically purified enzyme is initially inactive in a H₂-uptake assay. Only after the addition of a small amount of NADH (5 μM) it is reduced, and capable of oxidising H₂. When NAD⁺ is used as electron acceptor the specific activity is 60-80 U/mg (Schneider, 1979; Erkens, 1996). As mentioned before, the activity of the enzyme is only slightly hampered by oxygen. Under a partial oxygen pressure of 60% (0.7 mM O₂) the activity decreases less than 20% (Schneider, 1979). Carbon monoxide, a common inhibitor of [NiFe] hydrogenases, has no effect at all on the activity of the enzyme (Schneider, 1979).
1.2.2 Spectroscopic properties

After aerobic purification the SH is in an oxidised, EPR silent state. Sometimes at low temperatures (<20K) a small signal around $g = 2$ is observed (Schneider, 1979), but this represents only a very small percentage of the enzyme molecules and is most probably due to an oxidatively damaged [4Fe-4S] cluster. The active site, however, remains completely silent, contrary to observations in oxidised standard hydrogenases where an EPR spectrum attributed to a Ni-based unpaired electron can be observed (Albracht, 1982).

Upon reduction with hydrogen the Fe-S clusters become EPR detectable at low temperatures (Schneider, 1979). Incubation with excess NADH (5 to 10 mM) displays a signal from an unpaired electron based on a Ni site, very similar to the Ni$_{-}$C$^\prime$ state in standard [NiFe] hydrogenases (Schneider, 1996) that also displays the characteristic light sensitivity (Van der Zwaan, 1985). Optimisation and quantification of the signal, however, showed that only 0.24 spins per Ni ion could be detected (Happe, 2000).

The SH from R. eutropha is EPR silent in most states. It is therefore fortunate that, like standard [NiFe] hydrogenases, it shows absorption bands in the 2100-1900 cm$^{-1}$ region of the infrared spectrum (Happe, 2000). The frequencies of these absorption bands hardly responded to changes in the redox state of the enzyme, though. Similar to the assignments made in standard hydrogenases, the more intense band was assigned to an Fe coordinated CO molecule. The pattern in the cyanide region is somewhat more complicated and seems to correspond to four (partly overlapping) absorption bands (Happe, 2000). Since a quantitative chemical analysis yielded 4 CN$^{-}$ ligands per Ni (W. Roseboom and S.P.J. Albracht, unpublished result) and only one of the CN$^{-}$ absorption bands responded to reduction of the enzyme, the model as presented in Figure 9 was proposed for the active site.

![Figure 9. Active site of the O$_2$-tolerant [NiFe] hydrogenase from R. eutropha. Reprinted with permission from Happe, FEBS Lett, 466, 259-263. 2000. Copyright 2000, Federation of the European Biochemical Societies.](image-url)
X-ray absorption spectroscopy has been applied to study the properties of the EPR-silent Ni centre in SH of *R. eutropha* (Figure 10). The observed Ni K-edge of SH in the as-isolated, oxidised state differs considerably from the Ni-edges measured in standard [NiFe] hydrogenases (*Gu*, 1996; *Müller*, 1997). The energy at half-height of the absorption edge is 8,341 eV, indicative of a divalent Ni centre, and the small pre-edge features together with the edge-structure suggest a distorted octahedral coordination of nickel. Reduction of the *R. eutropha* SH with excess NADH (10 mM) brings about large changes in the Ni coordination (*Gu*, 1996; *Müller*, 1997). This change in ligand environment is also reflected in the EXAFS analysis of the XAS spectra (*Gu*, 1996; *Müller*, 1997).
1.3 [NiFe] hydrogenases that ‘sense’ hydrogen

A very special [NiFe] hydrogenase has been identified in *R. eutropha* (*Lenz, 1997*). The function of this hydrogenase is to sense *H₂* in the bacterial cell (*Kleihues, 2000*). If *H₂* is sensed, a signal is transmitted via a regulatory system and ultimately *H₂*-consuming hydrogenases are transcribed (*Lenz, 1998*).

Similar gas-sensing systems that convert a molecular signal into a cellular response have been described for *O₂*, *CO* and *NO* (for an overview see *Chan, 2001*). One example of an *O₂*-sensing protein is a heme-containing histidine protein kinase. The binding of the *O₂* molecule to the heme group induces a high-spin to low-spin transition of the Fe³⁺ ion, which initiates signal transduction. Also in the cases of *CO* and *NO* sensing the binding of the reporter molecule to a heme group, located in a PAS domain in the gas-sensing protein, initiates signal transduction. A different system for the sensing of *O₂* has been identified in *E. coli* where an Fe-S cluster conversion is the initiating step in signal transduction. *O₂* induces the conversion of a [4Fe-4S]²⁺ cluster into a [2Fe-2S]²⁺ cluster (*Kiley, 1999*).

In this part, a recently discovered regulatory system for *H₂* in *R. eutropha* is described. As it turns out, the protein used to initially detect *H₂* is structurally very similar to standard *H₂*-oxidising hydrogenases.

1.3.1 *H₂*-signal transduction

Hydrogenase-gene expression in *R. eutropha* is regulated by a *H₂*-sensing multi-component regulatory system (Figure 11). One constituent of this regulatory system is a [NiFe] hydrogenase, designated a regulatory hydrogenase (RH) (*Lenz, 1998*). The RH is functional as a dimer, (RH)₂, and the first step in the signal transduction is the binding of *H₂* to its active site. The (RH)₂ complex interacts with a two-component regulatory system consisting of a histidine protein kinase (HoxJ) and a response regulator (HoxA). The precise nature of this interaction is still unclear but it was recognised that HoxJ contained a PAS domain, a domain predominantly found in proteins involved in signal transduction (*Kleihues, 2000*). HoxA is negatively regulated by HoxJ, preventing hydrogenase gene expression in the absence of *H₂*. In the presence of *H₂*, HoxJ is inactivated by the dephosphorylation of the His220 residue, which concomitantly results in the dephosphorylation of the Asp55 residue of the response regulator HoxA. Since the HoxA in its dephosphorylated form activates transcription, this ceases the negative regulation by HoxA and transcription of the hydrogenase genes can occur. The hydrogen-sensing apparatus in *R. eutropha* has recently been reviewed in (*Lenz, 2002*).

![Figure 11. Model of the *H₂*-dependent signal transduction in *R. eutropha*. The figure is reprinted from *Lenz, 1998*. Details are described in the text.](image-url)
1.3.2 Properties of the Regulatory Hydrogenase

Homologues of the RH in *R. eutropha* have been identified in *Bradyrhizobium japonicum* (Black, 1996) and *Rhodobacter capsulatus* (Elsen, 1996). The RH protein of *R. eutropha* is a dimer consisting of a large subunit (HoxC, 52.4 kD) and a small subunit (HoxB, 36.5 kD). The primary structure contains almost all conserved signatures for metal coordination in [NiFe] hydrogenases. For the RH of *R. eutropha* the enzymatic and regulatory activity is shown to be dependent on Ni (Kleihues, 2000). Aside from these similarities to standard hydrogenases, the RH sequences display characteristics specific for regulatory hydrogenases. The lack of an N-terminal signal sequence to the small HoxB subunit indicates a cytoplasmic localisation of the RH. The large subunit is also devoid of a C-terminal extension. The small subunit contains a C-terminal extension of 54 to 55 amino acids including a stretch of 22 hydrophobic residues absent in standard hydrogenases. It seems as if this C-terminal domain is involved in linking the RH to its specific partners in signal transduction (Kleihues, 2000). Furthermore, all regulatory hydrogenases contain an insertion of 10 residues in the small subunit (Kleihues, 2000).

An initial spectroscopic analysis of the *R. eutropha* RH was performed on crude extract from cells overproducing RH in a strain lacking both the MBH and the SH (Pierik, 1998). The amount of RH in the cell increased to such an extent that spectroscopic detection became possible. The sample was essentially EPR silent in the aerobic, oxidised state. After exposure of the crude extract to H₂, a Ni₄-C signal was observed in EPR spectra (Pierik, 1998). FTIR spectra of the crude extract showed that the RH can occur in two redox states: an oxidised state (ν(CO) at 1943 cm⁻¹) similar to the Ni₄-S state, and a reduced state (ν(CO) at 1962 cm⁻¹) that resembles the Ni₄-C⁻ state. Contrary to standard hydrogenases, the RH could not be reduced any further than the Ni₄-C⁻ state, not could it be oxidised beyond the Ni₄-S state.

Activity measurements showed that the aerobic RH was able to immediately oxidise molecular hydrogen using a redox dye as electron acceptor (Pierik, 1998). Also after addition of oxidising agents, immediate H₂-oxidation occurred which indicates that the RH remains in an active state. The RH from *R. capsulatus* was shown to be able to bind H₂ and showed HD-exchange activity (Vignais, 1997, 2000). However, the observed activities are two orders of magnitudes lower than observed in standard [NiFe] hydrogenases (Pierik, 1998; Vignais, 2000).
1.4 Maturation of [NiFe] hydrogenases

The assembly of [NiFe] hydrogenase is a complex process. Mature hydrogenases contain an active site that holds two metal ions that bind several diatomic, non-protein ligands. In addition to this active centre, they usually also harbour several Fe-S clusters to conduct electrons at or from the active site. To assemble this rather complex structure consisting of protein, metal-cofactors and small organic ligands, a set of helper proteins is required. These proteins are involved in processes like metal storage, active-site assembly and proteolytic processing. The properties of these helper proteins, as well as their role in the assembly of the active site, are described in this part. The maturation of [NiFe] hydrogenases has recently been reviewed in (Casalot, 2001).

Instead of in the cytoplasm, some [NiFe] hydrogenases exert their activity in the periplasm. To reach the site of physiological function, they must be translocated over the cytosolic membrane. It turns out that the system involved in this process, the TAT system, is able to translocate fully folded and functional proteins over the cytosolic membrane. This remarkable accomplishment is described in the last part of this section.

1.4.1 The gene products involved in active-site assembly and their putative functions

Several accessory genes involved in the maturation of [NiFe] hydrogenase have been identified in hydrogenase-containing bacteria like E. coli (Lutz, 1991), Rhizobium leguminosarum (Hidalgo, 1992; Rey, 1993) and R. eutropha (Dernedde, 1993). The two hydrogenase operons on the 450-kb megaplasmid pHG-1 of R. eutropha are depicted in Figure 12. These operons contain the structural genes that encode the MBH, the RH and the SH [NiFe] hydrogenases, a cassette of hyp genes and a number of genes functional in regulation. The hyp genes are probably co-transcribed and their products are involved in the maturation and processing of all hydrogenases. Seven open reading frames are designated hypA1, hypB1, hypF1, hypC, hypD, hypE and hypX (Dernedde, 1993, 1996). In R. eutropha, three of the hyp genes are duplicated and named hypA2, hypB2 and hypF2 (Wolf, 1998). Most hyp genes are conserved in [NiFe] hydrogenase-containing bacteria and exert a pleiotropic effect in strains producing multiple hydrogenases (Jacobi, 1992; Dernedde, 1996a; Wolf, 1998). The functions of most Hyp proteins in [NiFe] hydrogenase maturation are still unclear. In this part the properties of the Hyp proteins as currently known are presented.

All Hyp proteins are soluble and located in the cytoplasm. The HypA1 protein (110 amino acids; all protein sizes mentioned here are from the Hyp proteins of R. eutropha, from the study by Dernedde, 1996a, unless mentioned otherwise) is a rather small and cysteine-rich protein. Four conserved cysteines are arranged into two Cys-X2-Cys motifs (X = any amino acid), suggesting a binding site for an Fe-S cluster. The HypB1 protein (361 aa) carries an N-terminus rich in histidines, suggested to bind divalent metal-ions (Dernedde, 1996a). A HypB homologue in E. coli was shown to contain a site for GTP hydrolysis in the C-terminus (Maier, 1993), an activity required for Ni-insertion into [NiFe] hydrogenase (Maier, 1995). A deletion of the hypB gene resulted in the formation of Ni-free hydrogenase precursors (Dernedde, 1996a). The small HypC protein (90 aa) binds to the precursor of the large subunit and is required for the incorporation of Ni (Dernedde, 1996a) although it contains
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no obvious metal-binding motif itself (Jacobi, 1992). HypD (379 aa) contains a Cys-X-Cys-X-Cys-X-Cys motif, and is therefore supposed to bind an Fe-S cluster (Dernedde, 1996a). Its role in the maturation process is not yet understood. Deletion of HypE (351 aa) also results in a complete blockage of the maturation process (Dernedde, 1996a). The HypF protein (394 aa) is absolutely required for [NiFe] hydrogenase maturation (Dernedde, 1996a). A recent study shows that HypF in E. coli is functional in the incorporation of CO and CN⁻ and that carbamoyl phosphate is a likely precursor molecule for these ligands (Paschos, 2001). HypF contains a consensus pattern of acyl-phosphatases (Wolf, 1998) and a motif present in proteins that catalyse O-carbamoylations (Paschos, 2001). The HypX protein (597 aa, Lenz, 1994) has thus far only been found in (facultative) aerobic bacteria and has been proposed to participate in the generation and transport of the CO and CN⁻ ligands (Rey, 1996). Sequence comparison showed that HypX contains an N-terminal tetrahydrofolate (THF) binding domain and it is therefore thought to play a role in the transfer of one-carbon species (Rey, 1996). In addition, the C-terminus of HypX shows homologies to an enoyl-CoA hydratase/isomerase. Nonetheless, a hypX deletion only results in decreased hydrogenase activity and is not lethal (Rey, 1996; Buhrke, 1998). As mentioned before, R. eutropha contains duplicates of three of the accessory proteins. The hypA2, hypB2 and hypF2 are located downstream the SH structural genes (Figure 12). These duplicates physiologically compensate for each other (Wolf, 1998).

Figure 12. Hydrogenase gene arrangement on the megaplasmid pHG1 of R. eutropha. Hydrogenase structural genes (hex) are shown in black, genes involved in metallocenter assembly (hyp) are shown in dotted boxes. Dr. O. Lenz is acknowledged for the preparation of this figure.

1.4.2 Model for active-site assembly

In the introductory part of this section it was already mentioned that the maturation of [NiFe] hydrogenase is a complex process in which all of the accessory proteins described before play their role. A cartoon of the maturation process in E. coli is presented in Figure 13. The earliest identified step in the maturation is the interaction of the precursor of the large subunit (pre-HycE in Figure 13) with HypC (Drapal, 1998). In E. coli this complex forms in all hyp deletion mutants other than the hypC negative mutant (Magalon, 2000b). Site-directed mutagenesis experiments showed that the Ni-binding Cys241 residue from the large subunit and the Cys2 residue of HypC are involved in the formation of the pre-HycE-HypC complex (Magalon, 2000b). The likely idea is that HypC acts as a chaperone in preventing the large-subunit precursor to incorporate Ni and fold properly. The pre-HycE-HypC complex has been shown to incorporate Ni as a late step in the maturation process (Magalon, 2000a). Before Ni-incorporation, the Fe-part of the active site is built and incorporated (Maier, 1996a). Recent evidence indicates that carbamoyl phosphate is a precursor molecule for the
diatomic ligands and that HypF plays an important role in the biosynthesis of the CO and CN ligands (Paschos, 2001). Since HypE, like HypF, is absolutely essential for maturation and the two proteins have been shown to interact (Rain, 1994), HypE might assist HypF in this step of the maturation. After the incorporation of the Fe atom, for which no mechanism is yet proposed, the Ni ion is delivered to the pre-HycE-HypC complex by the Ni-binding auxiliary protein HypB. A deletion of HypB results in the production of Ni-free precursors (Dernedde, 1996a).

The insertion of Ni into the precursor is driven by GTP hydrolysis (Maier, 1995). Only after the insertion of Ni into the protein the interaction between the large-subunit precursor and HypC is broken, and the Cys241 residue becomes available for the coordination of Ni (Magalon, 2000a). The final step in the maturation of [NiFe] hydrogenases is the proteolytic cleavage of the C-terminus of the large-subunit precursor by a specific endopeptidase (Menon, 1993; Rossmann, 1994). The endopeptidases involved in the final processing step of the R. eutropha SH and MBH are HoxW and HoxM, respectively. The cleavage of the C-terminal domain from the large subunit is essential for the proper formation of a mature hydrogenase (Massanz, 1997).

The C-terminus seems to prevent the association of the small and the large subunit until the maturation of the large subunit is finished. After proteolysis, the large subunit is allowed to fold to its final conformation, and to associate with the small subunit. If the final destination of the mature hydrogenase is the membrane or the periplasm it is targeted to these locations by an N-terminal signal sequence attached to the small subunit.

Figure 13. Model for the maturation of the large subunit of [NiFe] hydrogenases. The nomenclature of E. coli hydrogenases is used in this picture. Details are described in the text. Reprinted with permission from Casalot, Trends Microbiol. 9, 228-237. Copyright 2001, Elsevier Science.
1.4.3 Translocation of periplasmic [NiFe] hydrogenases

[NiFe] hydrogenases exert their activities at different locations in bacterial cells. Some [NiFe] hydrogenases are active in the cytosol of the bacterial cell; examples are the SH and the RH from *R. eutropha* (Schneider, 1976) and the SH from *Nocardia opaca* (Schneider, 1984). Many membrane-bound hydrogenases (MBH) are linked to the membrane via a b-type cytochrome as shown for the MBH of *R. eutropha* (Bernhard, 1997). Through this cytochrome the electrons, produced in the process of H₂-oxidation, are transferred to the electron-transfer chain in the membrane. The *R. eutropha* MBH is active in the periplasmic space (Schink, 1979). Other examples of periplasmic MBHs include the [NiFe] hydrogenases 1 and 2 from *E. coli* (Menon, 1990, 1994). An example of a soluble periplasmic hydrogenase is the standard [NiFe] hydrogenase from *D. gigas* (Li, 1987).

In order to function properly, these enzymes do not only require proper assembly and processing, but they also need to be targeted towards and across the cytosolic membrane. Translocation of a soluble protein, a rather hydrophilic moiety, across a hydrophobic lipid bilayer requires a lot of energy. The most common way to translocate proteins across the cytosolic membrane is through a set of proteins that cooperate in a secretion machinery, the so-called Sec machinery (for a review see Driessen, 1994). An essential feature of this translocation pathway is that the preprotein is translocated as a chain, that is, in an unfolded manner. This implies that the preprotein cannot bind any cofactors during translocation by the Sec-machinery as this usually requires (partial) folding. Thus, in this case further processing of proteins containing prosthetic groups must occur after translocation. It was recognised that a lot of periplasmic cofactor-containing proteins contain a signal sequence different from the Sec-pathway signal sequences (Berks, 1996). They are synthesised as precursors with amino-terminal signal sequences that are much longer than those that target to the Sec-pathway and harbour the distinctive (Ser/Thr)-Arg-Arg-X-Phe-Leu-Lys motif, the so-called twin-arginine sequence. It was shown that B-lactamase fused with a mutated signal sequence of the [NiFe] hydrogenase from *D. vulgaris* (the first conserved Arg was mutated to Glu) was impaired in translocation (Nivière, 1992). According to the authors, the Arg → Glu mutation is not expected to impair translocation via the Sec-system, indicating that another export pathway must be involved. This experiment for the first time illustrated the importance of this double-arginine conserved motif.

The machinery that is able to translocate precursor proteins that contain this motif is designated the twin-arginine translocation or TAT machinery. In *E. coli* four genes (*tatA, tatB, tatC* and *tatE*) are identified that encode components of the TAT translocase (Sargent, 2001) and all four components are associated to the membrane (Berks, 2000). The group of cofactor-containing proteins that require the TAT system for translocation is very divers. They may contain cofactors like various Fe-S clusters ([2Fe-2S] or [4Fe-4S] clusters), metal sites (Ni, Cu, Mo) or organic cofactors like flavins or quinones (Berks, 1996).

Since the small subunit of periplasmic [NiFe] hydrogenases is synthesised carrying a twin-arginine signal sequence, it was predicted that they also would use the TAT system for translocation. Indeed, replacement of the essential Arg residues for Glu residues resulted in the blockage of the translocation of the periplasmic membrane-bound [NiFe] hydrogenase of *Wolinella succinogenes* (Gross, 1999). The catalytically active, unprocessed precursor of the large subunit and the processed small subunit both
accumulated in the cytosol. This indicated that translocation of both the small and the large subunit depends on the TAT system (Gross, 1999). Similarly, the preprotein (still containing the signal sequence) of the membrane-bound [NiFe] hydrogenase from a R. eutropha strain impaired in the translocation of TAT-dependent proteins, was detected in the cytoplasm in an active form (Bernhard, 2000). Heterologous expression of the tatA gene from Azotobacter chroococcum restored the transport over the membrane of the MBH (Bernhard, 2000). Furthermore it is shown that the protein complex of the small and the large subunit is targeted to the TAT system by the N-terminal signal sequence of the small subunit and that both subunits are translocated simultaneously (Rodrigue, 1999). All these results suggest that periplasmic [NiFe] hydrogenases have their cofactors incorporated prior to translocation and that they are translocated in a fully folded, enzymatically active form.

1.5 Outline of this thesis

As mentioned before, different types of [NiFe] hydrogenases are described in this thesis. These metalloproteins all contain similar active centres with different metal atoms: one Ni and one Fe. Furthermore they contain several Fe-S clusters that function as an 'electrical wire', transporting electrons from the active centre to the surface of the molecule where the electrons are passed on to an electron-accepting partner molecule. In the first chapter an overview of the literature on [NiFe] hydrogenase research is given. The subject of the second and third chapter is the [NiFe] hydrogenase from the purple photosynthetic bacterium A. vinosum. This hydrogenase can only oxidise hydrogen gas if no oxygen is present. In the presence of oxygen it reversibly inactivates. The process of (in)activation is studied in chapter 2, using FTIR spectroscopy. In chapter 3, the nature of the ligand that blocks activity in the oxidised states is examined in more detail. A change in spectroscopic properties is described when oxidative inactivation in H₂O and D₂O is compared. In the following chapter the A. vinosum MBH is oxidised in an unorthodox way. The resulting state, though inactive, shows spectroscopic similarities with enzyme in an active state. From these similarities the redox state and the coordination of the Ni site in active enzyme are inferred.

The remaining part of this thesis is devoted to [NiFe] hydrogenases from the bacterium R. eutropha. This is a 'Knallgas' bacterium, which means that it can use a mixture of H₂ and O₂ as an energy source. Therefore, the hydrogenases it contains for the oxidation of H₂ must be O₂ tolerant. Chapter 5 describes how the active site from the soluble hydrogenase (SH) from R. eutropha is protected from oxygen. A helper protein is identified that modifies the active site as to prevent inactivation of the SH under aerobic conditions. R. eutropha contains yet another hydrogenase, a regulatory hydrogenase (RH). If this RH senses low concentrations of hydrogen gas, it generates a signal that induces the expression of H₂-oxidising hydrogenases in the cell. This allows the bacterium to use H₂ as a source of reducing equivalents, required for its metabolism. Chapter 6 describes how the RH was purified and characterised. The synthesis and maturation of a protein that contains so many cofactors is a complex phenomenon. In chapter 7 the proteins involved in the active-site assembly are described and their role in the maturation of the RH is studied. Mutants impaired in the functions of the helper proteins are constructed and the ability of these mutants to produce intact RH molecules is studied. Finally, a summary of the described work is presented in chapter 8.
General introduction to [NiFe] hydrogenases