

Hydrogenases from the Hyperthermophilic Archaeon

Pyrococcus furiosus

Proefschrift

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Chapter 1

General Introduction

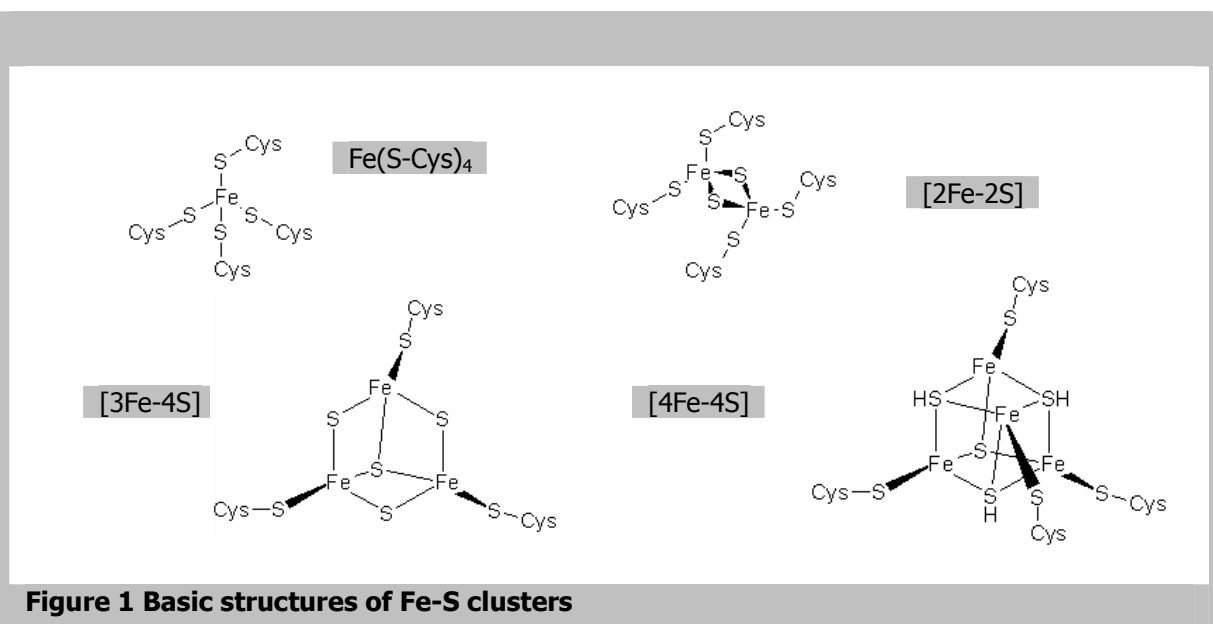
Introduction

Electron-transfer proteins

Electron-transfer (redox) reactions have been a continuing source of fascination to chemists and have proved to be central to the development of modern coordination chemistry. It should therefore come as no surprise that redox proteins and enzymes are among the best characterized and most widely studied by inorganic biochemists [1].

One of the groups of electron-transfer metalloproteins are the iron-sulfur proteins. Their primary function lies in mediating one-electron redox processes and as such they are integral components of respiratory and photosynthetic electron transfer chains and a host of redox enzymes and proteins involved with carbon, oxygen, hydrogen, sulfur and nitrogen metabolism. They also constitute in whole or in part the substrate-binding sites of a wide range of redox and nonredox enzymes and their key role in determining protein structure makes them important regulatory targets for controlling enzyme activity and gene expression in response to external stimuli such as intracellular Fe, NO, O₂ or O₂⁻ concentrations [2].

The iron-sulfur proteins exhibit four Fe-S clusters, i.e. Fe(S-Cys)₄, [2Fe-2S] clusters, cubical [3Fe-4S] clusters, and cubane [4Fe-4S] clusters with the [4Fe-4S] being the most frequently encountered cluster (Figure 1). The [3Fe-4S] cluster is found more rarely in nature. It is a variant of the [4Fe-4S] cluster with one iron plucked out of one corner [3, 4].



The active sites of a significant fraction of all proteins, including redox proteins and enzymes, contain one or more metal ions, conditioned by protein structure and environment towards accomplishment of evolutionary directed function. Their structural and electronic properties are often modulated from those of small molecules containing the same metal ion [3].

The ligands in electron-transfer proteins assist electron transfer either by reducing the charge on the metal ion or by inducing a low-spin, rather than high-spin, form of the metal. Such obliging ligands include sulfur, imidazole, and porphyrins. An essential feature of such electron-transfer metalloproteins is a strong firm protein fold that can accommodate a change in charge on the metal ion without much change in the ligand geometry around that metal. In such metalloproteins the metal ions are buried inside the protein and the coordination sphere is complete. As a result of this protective shield, small ions and water are unable to reach the metal ion and interact with it. The protein must provide a very selective route by which electrons can enter and leave it. This is done by careful location of the metal site in the protein [5].

Hydrogenases

Introduction

Hydrogenase is an electron-transfer protein and catalyzes the simplest of chemical redox reactions, the reversible two-electron oxidation of molecular hydrogen in aerobic and anaerobic microorganisms according to the following equation [6]:



Although most, if not all, known hydrogenases can catalyze the reaction in either direction in vitro, they are usually committed to catalyze either hydrogen uptake or evolution in vivo, depending on the demands of the host organism [7].

Hydrogenases were first isolated from prokaryotes of the domain Bacteria [8] then from Archaea [9]. The latter domain comprises two kingdoms, the Euryarchaeota and the Crenarchaeota [10] many members of which rely on hydrogen as a main energy source. Finally, hydrogenases have also been found in subcellular organelles of eukaryotes, namely hydrogenosomes of protozoa [11, 12] and chloroplasts of green algae [13, 14]. These enzymes are thus present in all domains of life [7].

All hydrogenases contain metal atoms. Most hydrogenases contain nickel and besides nickel they all contain several iron atoms, most of the iron being organized in Fe-S clusters (Figure 1) [15-17]. The hydrogenases are divided into two major groups by their metal contents: NiFe-hydrogenases, which contain Ni and Fe, and Fe-hydrogenases, which possesses only Fe atoms [7]. There are also hydrogenases which have Se as ligand to Ni, and they are sometimes referred to as a separate class, although they can be surely considered as a subclass of NiFe-hydrogenases. Another possible classification of hydrogenases is based on their biological function: hydrogen production, hydrogen consumption and bidirectional hydrogenases [18, 19].

Fe-hydrogenases

Fe-hydrogenases, in which no other metal than iron can be detected are most often found in hydrogen producing microorganisms while NiFe-hydrogenases are generally found in hydrogen consuming microorganisms. The iron in Fe-hydrogenases is typically organized in two kinds of clusters. Part of the iron is found in the form of classical [4Fe-4S] clusters and, sometimes, in [2Fe-2S] clusters. The remaining iron is in a 6Fe cluster [20] that is proposed to be the active site and is called the “H-cluster” [21]. Unlike NiFe-hydrogenases, which are composed of at least two subunits, several Fe-hydrogenases are monomeric and consist of the catalytic subunit only. However, heterodimeric and heteromultimeric enzymes are also known [7].

Fe-hydrogenases are efficient users of a wide range of electron donors and acceptors. Fe-hydrogenases differ greatly in size and number of accessory domains. This might suggest that these domains determine the selectivity of the enzymes towards redox partners. In addition to a conserved domain of ca. 350 residues containing the active site, they most often

comprise additional domains which accommodate Fe-S clusters and which may altogether consist of more than 800 residues [7].

The first Fe-hydrogenase sequences revealed modular structures composed of previously known domains and of a unique and much conserved C-terminal domain. The latter was therefore assumed to contain the H-cluster, and was subsequently found to be present in all Fe-hydrogenase sequences. 3D structures have confirmed the modular structure of Fe-hydrogenases, and revealed the unique protein fold of the H-cluster domain, its high conservation, and its unprecedented metal cluster. Totally conserved amino acids include the four cysteine ligands of the active site, a few residues lining the active site cavity, as well as those suspected to be involved in the molecular hydrogen and proton channels connecting the buried active site to the surface of the protein [7].

NiFe-hydrogenases

NiFe-hydrogenases are generally found in hydrogen consuming microorganisms; in nitrogen fixers this hydrogen is produced by the organism itself in the N_2 -fixation reaction. NiFe-hydrogenases have been characterized from various organisms. The NiFe-hydrogenases are generally oxygen stable, i.e. reversibly inactivated [22]. Besides one nickel they all contain several iron atoms, most of the iron being organized in Fe-S clusters, at least one in the form of [4Fe-4S] cluster, a cubane structure. Some nickel containing hydrogenases contain selenium in the form of selenocysteine and are often called NiFeSe-hydrogenases [15, 16]. The selenocysteine residue in the NiFeSe-hydrogenases replaces the terminal cysteine residue corresponding to Cys 530 of the *Desulfovibrio gigas* enzyme in the first coordination sphere of nickel [23].

All NiFe-hydrogenases contain at least a small (around 30 kDa) subunit, which harbors a variable number of Fe-S clusters, and a larger (around 60 kDa) subunit where the active site, a cysteine-bridged dinuclear Ni-Fe cluster is located [24]. Other subunits are also present in some cases, allowing the hydrogenases to interact with dedicated electron carriers (NAD^+ , F_{420} , cytochrome b) [25].

The Active Site of Hydrogenases

Compared to most enzymes, which often undergo considerable conformational changes on binding of the substrates, hydrogenases are rather rigid proteins. Probably the only parts of the enzyme that move significantly are amino acid side-chains and bound water molecules, involved in transfer of protons to the active site [26]. Transport of H^+/H_2 to or from the active sites almost certainly takes place through specific channels in the protein matrix and the sites are 'wired' to the surface for electron exchange with their partner redox proteins by a conduit of Fe-S clusters, as illustrated for the Fe-hydrogenase from *Desulfovibrio desulfuricans*, Figure 2 [27].

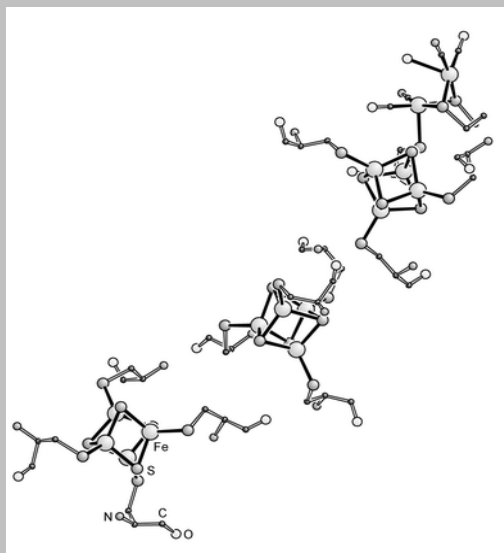
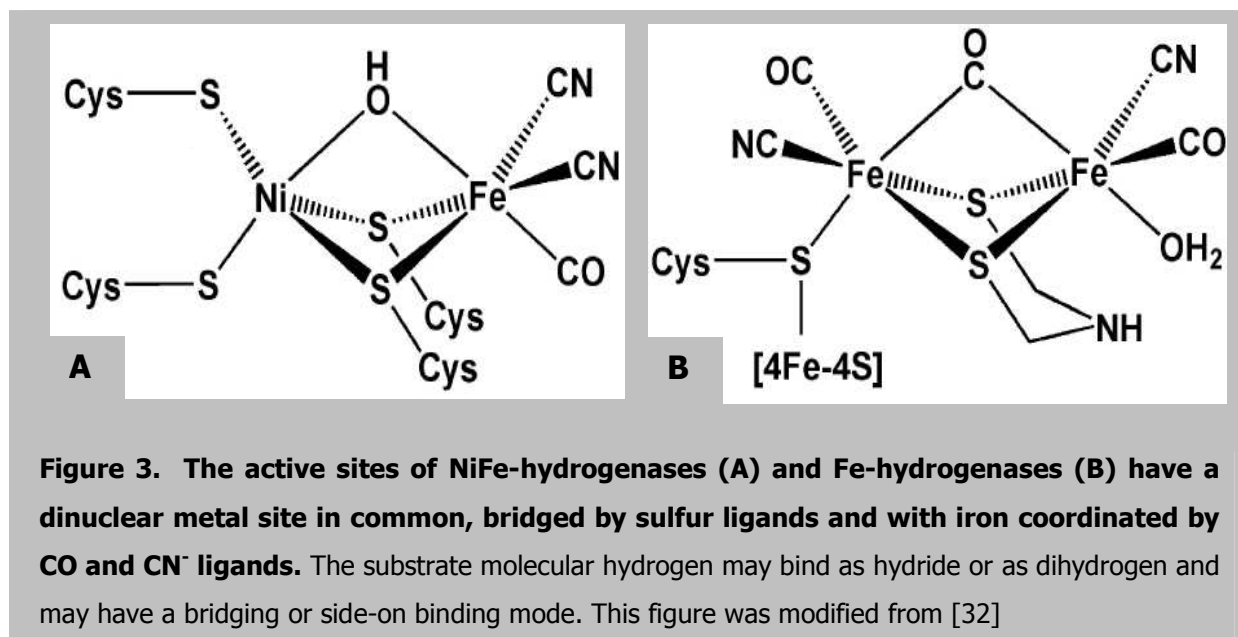


Figure 2 The Fe-S clusters of the Fe-hydrogenase from *Desulfovibrio desulfuricans*

In both NiFe- and Fe-hydrogenases the active sites are deeply buried within the protein matrix. For example, the NiFe-center in the hydrogenase from *D. gigas* is about 30 Å from the molecular surface [27]. The active sites of both Fe- and NiFe-hydrogenases contain CO and CN^- ligands which stabilize metals in low-oxidation states. In NiFe-hydrogenases, the dinuclear Ni-Fe center is bound to the protein through four cysteic sulfur ligands and four hydrogen bonds mediated by the two cyanide ligands [28]. By contrast, the dinuclear Fe-Fe center of Fe-hydrogenases is only bound to the protein through the thiolate ligand bridging between one of the Fe atoms and the closest [4Fe-4S] cluster and three hydrogen bonds involving the cyanide ligands.

As mentioned before, the Ni atom of the NiFe-hydrogenases is coordinated by four cysteine-sulfur atoms one of which is sometimes substituted by selenocysteine, as in the hydrogenase from *Desulfomicrobium baculatum* [29]. Two of the cysteine-sulfur atoms bridge to the Fe atom. There is an additional bridging feature, probably oxo or hydroxo, which is not present in the active form of the enzyme. The other ligands to Fe are diatomic cyanide and carbon monoxide (Figure 3) [30].

The active site cluster of Fe-hydrogenases, the ‘H cluster’, consists of a single [4Fe-4S] subcluster bridged through a cysteine S to a unique two-Fe subcluster. The [4Fe-4S] subcluster presumably functions in electron transfer, and the two-Fe-containing subcluster is the likely site of reversible hydrogen oxidation. The two-Fe subcluster consists of two Fe atoms that, in the case of the hydrogenase CpI from the anaerobic *Clostridium pasteurianum*, each exist in an octahedral coordination environment. Other ligands to the Fe atoms of the two-Fe subcluster in CpI are four diatomic ligands (one CO and one CN⁻ for each Fe atom), a terminal water molecule, two additional S atoms and one CO molecule that serve as bridging ligands between the two Fe atoms. The S atoms that bridge the two Fe atoms appear to be covalently linked to each other through a bridging moiety; the presumed composition of the bridge is ⁻S-CH₂-CH₂-CH₂-S⁻ or ⁻S-CH₂-NH-CH₂-S⁻ (Figure 3) [31].



Function of Hydrogenases

Although hydrogenases catalyze a very simple reaction, they do so in many different metabolic contexts and thus assume very diverse functions [8]. A specific microorganism may contain one or more different hydrogenases which are variously regulated in response to substrates and growth conditions [8, 21, 23]. The physiological functions are quite diverse, but can be grouped in three main functional areas. First: hydrogen consumption, second: hydrogen production and lastly: a combination of the two. Hydrogen consumption may be coupled to the reduction of different electron acceptors such as O_2 , NO_3^- , SO_4^{2-} , CO_2 and fumarate [33, 34]. In fermentative bacteria of the clostridial type, the reduction of protons to molecular hydrogen by hydrogenases is a means of disposing of excess reducing equivalents [8]. Hydrogen may be directly involved in proton translocation via the diffusion of hydrogen across the cytoplasmic membrane (hydrogen cycling or hydrogen recycling), and this requires hydrogen production on one side of the membrane and hydrogen consumption on the other side [33-38]. This bioenergetic process has been proposed to occur in some of the sulfate reducing bacteria and possibly other physiological types of bacteria including *E. coli* and acetogenic bacteria. Thus hydrogenase may play a unique role in biological energy conservation and represent an ancient mechanism for the generation of a proton gradient [22, 35, 39]. On the other hand, transmembrane hydrogenase-complexes are thought to directly pump protons across the membrane [40]. The transmembrane hydrogenase of *Methanosarcina barkeri* has been proposed to function as a proton pump when producing hydrogen during acetate-dependent growth [41]. The two reactions of hydrogenases can occur in different microorganisms and their coupling is the singular physiological characteristic underlying the concept of interspecies hydrogen transfer, which unites biochemical classes of anaerobic bacteria into consortia responsible for the conversion of cellulose and other organic substrates to methane and CO_2 [38, 42].

Kinetics

Michaelis-Menten

The following outline is based on the excellent text on enzyme kinetics by A. Cornish-Bowden [43]. Reaction velocity as a function of substrate concentration often follows the Michaelis-Menten equation:

$$v = \frac{V_{\max} [S]}{K_M + [S]} \quad \text{Equation 2}$$

It is the fundamental equation of enzyme kinetics. $V_{\max}$ and K_M are constants at a given temperature and pH. When the reaction follows this equation it is then said to display Michaelis-Menten kinetics. The term hyperbolic kinetics is also sometimes used because a plot of v against S has the form of a rectangular hyperbola through the origin (Figure 4).

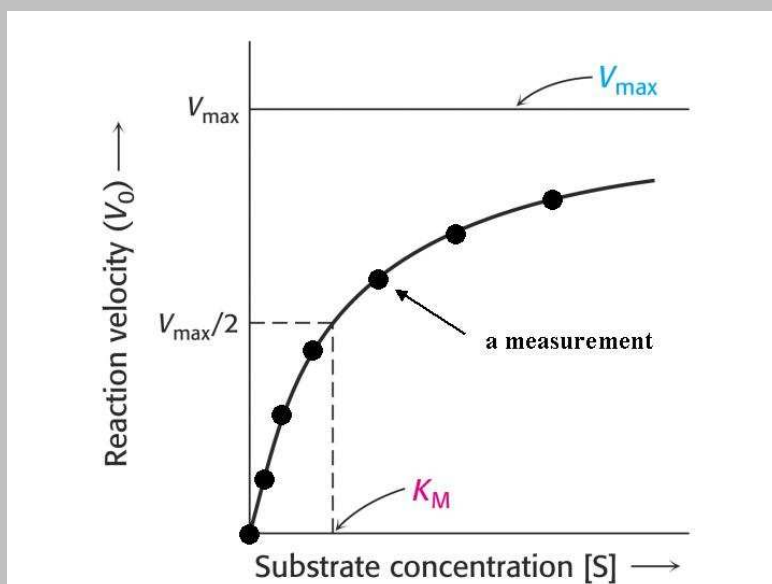


Figure 4 An example of a plot of initial reaction velocity against substrate concentration. The solid trace is a fit to the Michaelis-Menten equation

$V_{\max}$ (U/mg) is the limiting or maximal rate of an enzymatically catalyzed reaction. The substrate is in great excess, such that the enzyme is fully saturated as ES. The reaction rate $V_{\max}$ is dependant only upon the rates of the subsequent conversion of the bound substrate. K_M (M) is called the Michaelis constant and is the concentration of the substrate at which $v=0.5V_{\max}$. The ratio between the $V_{\max}$ and the total enzyme concentration is the first-order rate constant, k_{cat} (s^{-1}). k_{cat} is called the catalytic constant or the turnover number. It is the maximum number of moles of substrate (S) converted to product per unit time per mole of enzyme active site (since an enzyme may have more than one active site). To understand the meaning of K_M , we need to have a model of enzyme action. The simplest model is the classic model of Briggs and Haldane (1925):



and with $E = E_0 - ES$ (where E_0 is the total enzyme concentration) this leads to the following equation:

$$\frac{d[\text{ES}]}{dt} = k_1 ([E_0] - [\text{ES}]) [\text{S}] - k_{-1} [\text{ES}] - k_2 [\text{ES}] \quad \text{Equation 4}$$

Briggs and Haldane argued that a steady state would be reached in which the concentration of the intermediate was constant, with $d[\text{ES}] / dt = 0$. This assumption leads to the Michaelis-Menten Equation 2 for $k_2 = k_{\text{cat}}$ and $K_M = (k_{-1} + k_2) / k_1$.

Steady-state kinetics

The concept of steady state is widely used in dynamic systems. It generally refers to the situation in which the value of a particular quantity is constant, is “in a steady state”, because its rate of formation is balanced by its rate of destruction. For example, the concentration of a metabolite in a cell is at a steady-state level when it is being produced as rapidly as it is being degraded. In enzyme kinetics, the concept is applied to the

concentrations of enzyme-bound intermediates. When an enzyme is mixed with a large excess of substrate, there is an initial period, known as the pre-steady state, during which the concentrations of these intermediates build up their steady-state levels. It is during this steady-state period that the rates of enzyme reactions are traditionally measured. The steady state is an approximation, since the substrate is gradually depleted during the course of the experiment. But, provided that the rate measurements are restricted to a short time interval over which the concentration does not greatly change, it is a very good approximation [44].

Inhibition

A substance that decreases the rate of an enzyme-catalyzed reaction when it is present in the reaction mixture is called ‘inhibitor’. Inhibition can arise in a wide variety of ways and there are many different types of inhibitors [43].

Most elementary accounts of inhibition discuss two types of inhibition, i.e. competitive inhibition and uncompetitive inhibition. The competitive inhibition is so-called, because the substrate and the inhibitor compete for the same site. The mechanism may be represented in general terms as shown in Figure 5.

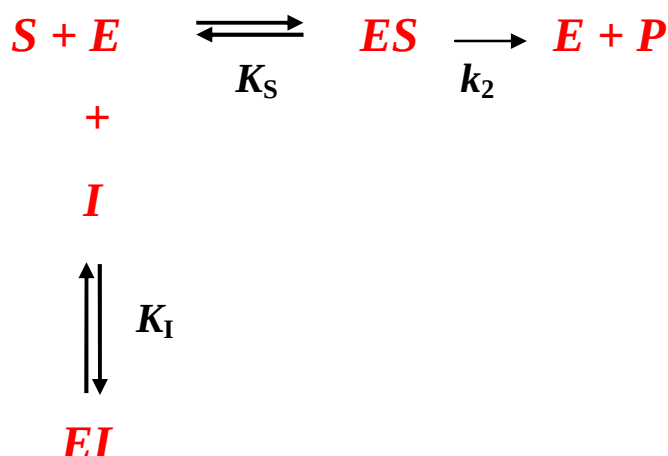


Figure 5. A mechanism of competitive inhibition

Uncompetitive inhibition is, at least in principle, the mechanistic converse of competitive inhibition, because it is predicted for mechanisms in which the inhibitor binds only to the enzyme-substrate complex and not to the free enzyme. This mechanism may be represented in general terms as shown in Figure 6.

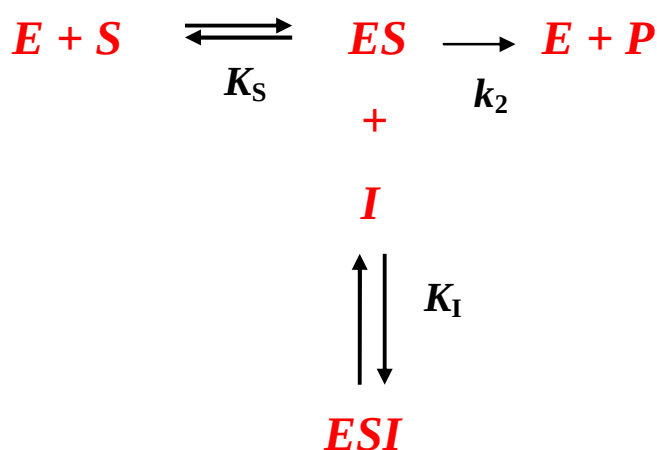


Figure 6. A mechanism of uncompetitive inhibition

Estimation of Michaelis-Menten parameters

Steady-state experiments are done by measuring (at chosen concentrations of enzyme (E), substrate (S) and inhibitor (I), pH, and temperature) the amount of product (P) formed or the amount of substrate reacted at one time, at several times or continuously. In the latter two cases the relation between P and time (t) is called the progress curve of the reaction. The data can then be treated in two quite different ways. The first is to calculate the initial velocity (v_0) of the reaction at $t=0$ at several concentrations of substrate, product or inhibitor and then examine the relationship between v and S, P or I. The second is to fit an integrated form of the Michaelis-Menten equation to the progress curve. Its big advantage is that (in the absence of complications of the progressive denaturation of the enzyme or its inhibition by the product) one can in theory calculate K_M and $V_{\max}$ from a single curve. Its disadvantage is that it is difficult to tell whether the Michaelis-Menten equation really does fit the data. Another

disadvantage is that the presence of relatively small errors in the data, either random or systematic, leads to relatively large errors in the estimates of K_M and $V_{\max}$.

Outline of this thesis

NiFe-hydrogenases are often associated with hydrogen consumption and Fe-hydrogenases are often associated with hydrogen production. Reported K_M values for hydrogen appear to be consistent with this classification where Fe-hydrogenases have higher values than NiFe-hydrogenases. However, this simple scheme leads to conflicts with physiological models. Therefore, we set out to re-determine the K_M of hydrogen oxidation for a NiFe-hydrogenase and a Fe-hydrogenase (Chapter 2).

While detailed structural knowledge of the hydrogenases is available, the mode and the locus of binding of the substrate, hydrogen, have not yet been established. A measurement of the hydrogen oxidation reaction by Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) revealed an inconsistency when analyzed as a Michaelis-Menten plot or in the form of a progress curve. Therefore, we undertook a detailed kinetic study of the activity/activation of this enzyme (Chapter 3).

The physiological role of the soluble hydrogenase(s) in *Pyrococcus furiosus* is not clear; the literature documents several subsequent re-assignments of putative function. We have therefore re-investigated the steady-state kinetics of the soluble hydrogenase in the *P. furiosus* (Chapter 4).

Previous analysis of the structural genes of *P. furiosus* hydrogenase-I suggested the presence of a large number of electron-transfer Fe-S clusters [45], where earlier EPR studies could only identify two or three clusters [46]. We re-investigated the EPR of this enzyme in a search for the “missing” clusters (Chapter 5).

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Chapter 2

***On the Relationship between the Affinity for
Molecular Hydrogen and the Physiological
Directionality of Hydrogenases***

Abstract

The physiological significance of the generic reaction $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$ is not always clear because hydrogenases may function in the breakdown of molecular hydrogen or in its synthesis or in both directions. Fe-hydrogenases have nevertheless been most often associated with proton reduction and NiFe-hydrogenases with hydrogen oxidation. A re-determination of the K_M of hydrogen oxidation by *Pyrococcus furiosus* NiFe-hydrogenase-I and by *Desulfovibrio vulgaris* Fe-hydrogenase suggests that affinity for hydrogen has been seriously underestimated and that the kinetics of hydrogen activation in relation to the directionality of hydrogenases should be re-evaluated.

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Introduction

Hydrogenases catalyze the generic reaction $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$. The conceptual simplicity of this reaction is misleading in view of the structural complexity and variability of the biocatalyst, of its biosynthetic machinery, and of its natural redox partners [1]. This complexity is also apparent from the variety of physiological functions ascribed to hydrogenase enzymes: (i) redox balancing, (ii) derivation of energy from hydrogen oxidation, (iii) production of reduced intermediates, e.g. NAD(P)H, (iv) proton respiration and/or (v) proton-gradient build-up. Furthermore, purified hydrogenases exhibit a wide range of activity ratios in assays for the production versus the consumption of molecular hydrogen. Three modifiers: ‘consumption’ (or ‘uptake’), ‘production’ and ‘bidirectional’ are used to indicate a bias (or its absence) in directional catalytic competence of hydrogenase, and thus to imply a specific physiological role. It is not always clear what the experimental basis for this interpretation is.

From early on it has been realized that the enzyme of the Fe-only type is concerned with hydrogen formation in, e.g., the metabolism of saccharolytic fermentative bacteria. Since then Fe-hydrogenases are generally associated with proton reduction, whereas NiFe-hydrogenases are most often associated with hydrogen oxidation [2]. Reported K_M values for hydrogen appear to be consistent with this classification where Fe-hydrogenases have higher values than NiFe-hydrogenases (Figure 1). However, this simple scheme leads to conflicts with physiological models in which Fe-hydrogenase should consume hydrogen (e.g. [3] and references cited therein) and with observations of thermophilic soluble NiFe-hydrogenases with an apparent preference for hydrogen production [4, 5].

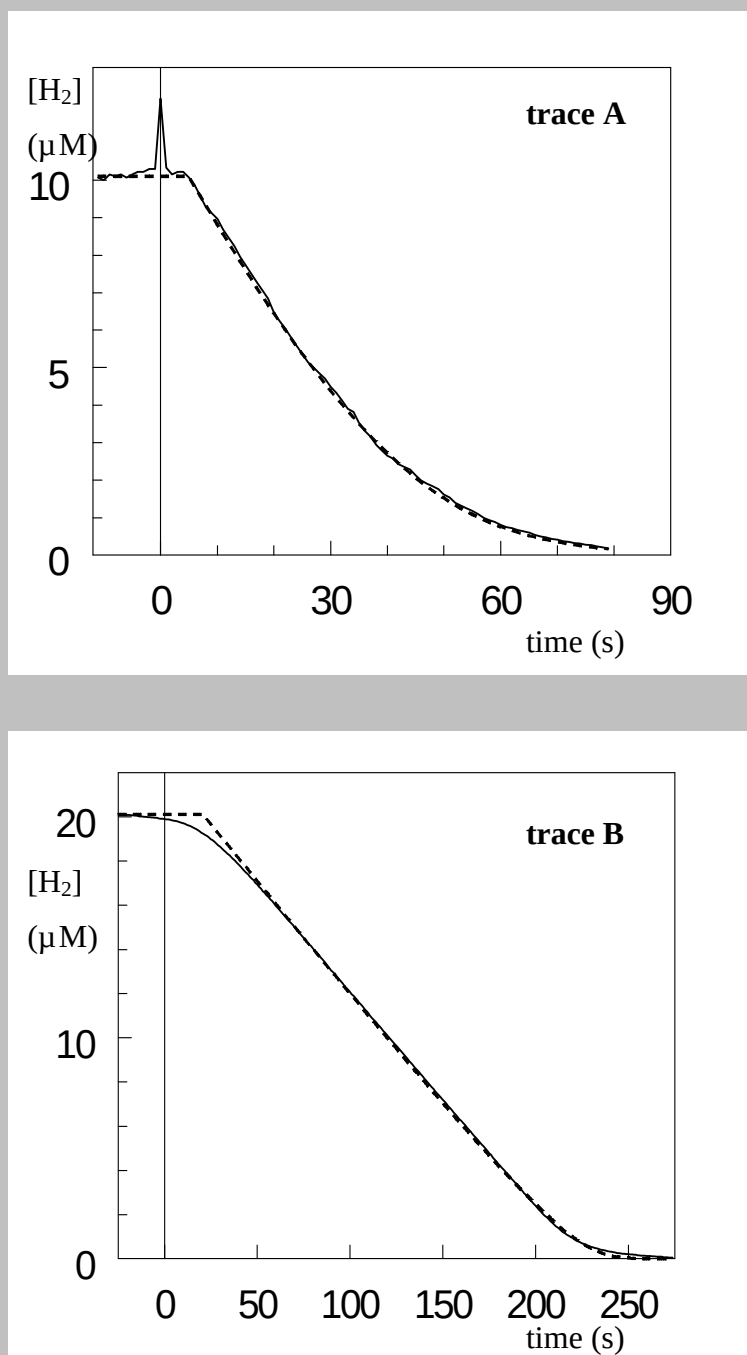


Figure 1. Progress-curve analysis of hydrogen oxidation by (trace A) *P. furiosus* (DSM 3638) hydrogenase [4, 6], and by (trace B) *D. vulgaris*, strain Hildenborough (NCIB 8303) Fe-hydrogenase [7].

Hydrogen consumption activity was measured with 1 mM methyl viologen as the artificial electron acceptor in 100 mM Epps buffer, pH 8.0 at 80 °C **(A)** or 30 °C **(B)**. H_2 was determined polarographically with a Clark-type electrode polarized at 600 mV versus Ag/AgCl at the assay temperature. The solid lines are fits with progress curves assuming simple, one-substrate Michaelis-Menten kinetics with $K_M (H_2) \approx 5 \mu M$ **(A)** or 1 μM **(B)**.

Overview of hydrogenase K_M (H_2) data

Table 1 gives an overview of K_M (H_2) data reported for NiFe- and Fe-hydrogenases in the period 1963-2000. K_M (H_2) values for NiFe-hydrogenases vary over three orders-of-magnitude from 0.6×10^{-7} to 0.4×10^{-4} M. The exception is *P. furiosus* whose soluble hydrogenase-I has been suggested to be ‘a new type of evolution hydrogenase’ [4]. With this enzyme excluded the range does not overlap with that reported for Fe-hydrogenases of 0.1×10^{-3} to 0.7×10^{-3} M. In the latter class, *Clostridium pasteurianum* hydrogenase-II is an exception; the physiological significance of this enzyme is a long-standing mystery [8].

Species	Type	K_M (μ M)
NiFe-hydrogenases		
- <i>Achromobacter ruhlandii</i>		8
- <i>Desulfovibrio vulgaris Miyazaki</i>		<8
- <i>Ralstonia eutropha</i>	(s)	37
	(mb)	32
- <i>Bradyrhizobium japonicum</i>		0.9-2.5
- <i>Paracoccus denitrificans</i>	(mb)	0.06-26
- <i>Azotobacter vinelandii</i>		0.86-1.3
- <i>Pyrococcus furiosus</i>	(sII)	130
	(sI)	140
Fe-hydrogenases		
- <i>Clostridium pasteurianum</i>	(I)	286-669
	(II)	32
- <i>Thermotoga maritima</i>		≈100
- <i>Megasphaera elsdenii</i>		170-269
- <i>Trichomonas vaginalis</i>		152

Table 1. K_M (H_2) values for NiFe-hydrogenases and Fe-hydrogenases. The type is soluble (s) or membrane-bound (mb). For some species two distinct enzymes (I,II) have been identified

Hydrogen oxidation by *Pyrococcus furiosus* NiFe-hydrogenase

This $\alpha\beta\gamma\delta$ heterotetrameric enzyme with a NiFe-hydrogenase part ($\alpha\delta$) and a diaphorase part ($\beta\gamma$) specific for NADPH [4-6] has previously been proposed to produce hydrogen from NADPH because it is more active in hydrogen production than in consumption and it has a high K_M (H_2) = 140 μ M [5]. In contrast, we have proposed the reverse activity of reducing $NADP^+$ with hydrogen to be the physiological relevant one because the bulk of hydrogen production can be accounted for by the production activity of a different, membrane-bound NiFe-hydrogenase complex [9].

In trace A of Figure 1 an example of an activity trace is given for the oxidation of hydrogen by the soluble hydrogenase-I. The results have been analyzed by progress-curve analysis, namely, by a fit to step-wise time-integrated Michaelis-Menten curve for one substrate assuming $K_M = 5 \mu$ M. This procedure was chosen instead of the conventional Lineweaver-Burk fit using initial rates because the latter are dependant on the number of activated hydrogenase molecules. Determination of the K_M from the progress curve is independent of the reproducibility of the extent of enzyme activation. The determined K_M is an order-of-magnitude less than the literature value [5].

Hydrogen oxidation by *Desulfovibrio vulgaris* Fe-hydrogenase

The hydrogen-production kinetics of the Fe-hydrogenase from *D. vulgaris* (Hildenborough) has been previously studied [10]. No pertinent results, in particular K_M -values, are found in the literature for hydrogen oxidation.

As illustrated with the activity trace in Figure 1(B), a $K_M \approx 1 \mu$ M for hydrogen has now been determined for the *D. vulgaris* (Hildenborough) enzyme using progress-curve analysis, and this is two orders-of-magnitude less than the values hitherto reported for ‘bidirectional’ or ‘production-type Fe-hydrogenases (compare with Table1). The number is within the range of values typical for NiFe-hydrogenases and would seem to suggest the Fe-

hydrogenase from *D. vulgaris* (Hildenborough) to be of the ‘uptake’ type, consistent with its proposed function in bioenergetics [3].

Need for a re-assessment of steady-state kinetic data

Most steady-state kinetic studies of hydrogenases (e.g. Table 1) were completed before crystal structures became available. The latter have unexpectedly revealed that the active sites of NiFe-hydrogenases and Fe-hydrogenases are similar in that they encompass a dinuclear metal site with cyanide and carbon monoxide ligands [11]. Any directionality is not obvious from the structures, and the question has not been addressed yet in subsequent quantum-chemical analysis. The dead time of available mixing equipment is currently too long to resolve primary reactions of hydrogenases [12, 13], and pre-steady-state kinetic studies have been focused on CO inhibition [13] or on redox activation/deactivation [14] with no reference to catalytic directionality. Direct electrochemical studies have been interpreted to indicate separate catalytic cycles for hydrogen oxidation versus proton reduction [15], however, the hydrogen oxidation was driven by a working electrode potential of +240 mV which is well over half-a-volt more positive than the midpoint potential of natural electron acceptors of hydrogenases. Finally, steady-state results have been collected using a variety of assay methods and conditions and enzymes at different stages of purification, which makes it a hazardous undertaking to compare literature values for basic entities, e.g. k_{cat} and K_{M} . to illustrate the putative softness of available results we have determined the K_{M} (H_2) for two hydrogenases to be at least an order-of-magnitude less than comparable literature values.

In the light of the previous studies it is proposed that steady-state kinetics of biological hydrogen activation in various species be carefully redetermined and reanalyzed preferably using purified enzymes under standardized conditions. This will provide a data set of reference for a subsequent endeavor into the fundamental and practical question of the relation between enzyme structure and directional function of hydrogenases.

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Chapter 3

***Biological Hydrogen Activation is Cooperative:
One H_2 Activates the Dinuclear Center and a
Second H_2 is Oxidized***

Abstract

Molecular hydrogen is activated by two classes of enzymes, Fe-hydrogenases and NiFe-hydrogenases, which have similar sulfur-bridged dinuclear metal active sites with biologically unusual ligands CO and CN⁻. While detailed structural knowledge on the biocatalyst is available, the mode and the locus of binding of the substrate, hydrogen, have not yet been established. A kinetic study of the hydrogen oxidation reaction by Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) reveals a biphasic activation mechanism from as isolated, resting enzyme, via an intermediate state of relatively low activity, to maximally active enzyme. Hydrogen itself is the causative agent for the two subsequent enzyme activation processes. Kinetic model analysis suggests that the steady-state assumption of Michaelis-Menten kinetics does not apply to this hydrogenase. It appears that activation by hydrogen is cooperative: a catalytic H₂ with high binding affinity is turned over at high rate only after a second, regulatory H₂ with low affinity has been bound to the active site. The proposed novel paradigm of two hydrogen molecules binding simultaneously to the active site may hold for hydrogenases in general.

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Introduction

The question “where does the substrate bind” is a recurring theme in the biological activation of diatomics such as H₂, CO, N₂, whose simple structures sharply contrast with that of their biocatalysts. These complex proteins have metal cluster active sites that are unusual to the extent that even high-resolution protein crystallographic data do not readily afford insight into the ways of binding interaction in enzyme-substrate intermediates. Long term attempts to determine the actual binding modes are typically based on a combination of data from crystallography, pre-steady-state kinetics, quantum-chemical calculations and multiple spectroscopies. A consensus view for the binding of molecular hydrogen to its enzyme, hydrogenase, is yet to emerge.

Hydrogenase catalyzes the simplest of chemical redox reactions:



This reaction has been suggested to be prototypical for metallo-enzymes insofar as its mechanism combines acid/base chemistry and electron transfer without involvement of an additional substrate [1]. The hydrogenases can be generally classified into two classes by their metal contents: the Fe-hydrogenases and the NiFe-hydrogenases [2]. There is no significant secondary or tertiary protein structure homology between the two classes, but the two types of active centers have structural properties in common, suggesting common mechanistic aspects (Figure 1).

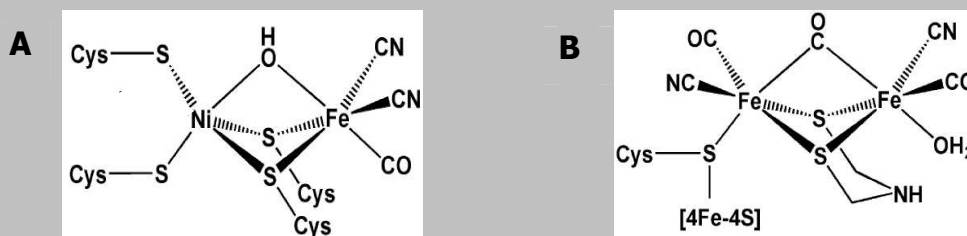


Figure 1. The active sites of NiFe-hydrogenases (A) and Fe-hydrogenases (B) have a dinuclear metal site in common, bridged by sulfur ligands and with iron coordinated by CO and CN⁻ ligands. The substrate molecular hydrogen may bind as hydride or as dihydrogen and may have a bridging or side-on binding mode. This figure was modified from [3]

Both are dinuclear metal clusters with bridging sulfur ligands and with presumably low-valent – low-spin iron coordinated by CN^- and CO ligands [4, 5]. The characterization of substrate binding has been hampered by several factors: (i) H is elusive in protein crystallography; (ii) the enzyme-substrate forming reaction is faster than the dead time of the mixing equipment [6, 7]; (iii) purified NiFe-hydrogenases, in particular, exhibit a complex activation mechanism with several intermediate states [2]. However, double-resonance data and/or DFT calculations have been the basis for specific, and partially conflicting proposals on side-on or bridging binding modes of hydride or molecular hydrogen to the metal(s) of the NiFe or FeFe active site (see, e.g., [3], and Refs therein and [8]). In all these proposals it is assumed that maximally only a single hydrogen/hydride interacts with the active site at any time. On the basis of a re-evaluation of steady-state kinetics we propose here a paradigm change: hydrogenase activity requires the binding of two separate hydrogen molecules: a positive modulator and a substrate.

Experimental Procedures

Source of enzyme

The sulfate-reducing, strictly anaerobic bacterium *D. vulgaris* subspecies Hildenborough (NCIB 8303) was grown batch wise on lactate, and its soluble, periplasmic Fe-hydrogenase was purified as described earlier [9].

Enzyme assay for methyl viologen reduction activity

Methyl viologen reduction activity was measured optically in a thermostatted glass fiber CCD array spectrophotometer (Avantes AVS-S2000), by the increase of absorption at 600 nm using $\epsilon_{602} = 9.7 \text{ mM}^{-1}\text{cm}^{-1}$ (or at 500 nm for high viologen concentrations; $\epsilon_{500} = 2.0 \text{ mM}^{-1}\text{cm}^{-1}$), at 32°C under anaerobic conditions in a 1 ml rubber-stoppered cuvette. The cuvette was filled with a given concentration of methyl viologen in 100 mM Epps, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid, pH 8.0, made anaerobic by repeated

vacuum/argon cycles on a vacuum manifold followed by slight pre-reduction of the viologen with sodium dithionite until a faint blue color would persist, and saturated with hydrogen by bubbling. The reaction was initiated by anaerobic addition of enzyme under argon. One unit of activity is defined as two μmol of methyl viologen reduced to the semiquinone radical per minute.

Enzyme assay for hydrogen consumption activity

Hydrogen consumption activity was measured amperometrically under anaerobic conditions (prolonged bubbling with argon followed by slight pre-reduction of viologen with sodium dithionite) with a Clark-type electrode (YSI 5331) polarized at 600 mV versus Ag/AgCl in a 2 ml water-jacketed cell. The temperature was held at 32°C by circulating water. The dual electrode was interfaced to a PC via a National Instruments 16 bits ATD converter, and current data were collected with a Lab-View program at 0.1 s intervals. The assay mixture was 1 mM methyl viologen in 1 ml 100 mM Epps, pH 8.0, and variable concentration of hydrogen obtained by dilution with a solution saturated with hydrogen. The solubility of hydrogen at 32°C is 763 μM . The reaction was initiated by anaerobic addition of enzyme under argon. One unit of enzyme activity is defined as one μmol hydrogen oxidized per minute.

Results and Discussion

K_M for methyl viologen in the hydrogen oxidation reaction

Methyl viologen (MV) is used as an artificial electron acceptor, therefore the generic reaction in Equation 1 becomes:



in which MV_{semi} is the colored, one-electron reduced semiquinone radical. Upon initiation of the reaction by addition of enzyme a lag phase is observed of circa 90 s over which the Fe-hydrogenase is apparently converted from a resting state to active catalyst (denoted as ‘intermediate’; see below). Therefore, the methyl viologen reduction activity was determined after circa 90 seconds, i.e. at maximal and constant rate, and the corresponding substrate concentration was read out from the abscissa of the activity trace (Figure 2). This rate after activation, v_{inter} , is plotted versus substrate concentration in Figure 3 and the data are fitted to the Michaelis-Menten equation:

$$v_{\text{inter}} = V_{\text{max}} \cdot [MV] / (K_M + [MV]) \quad \text{Equation 3}$$

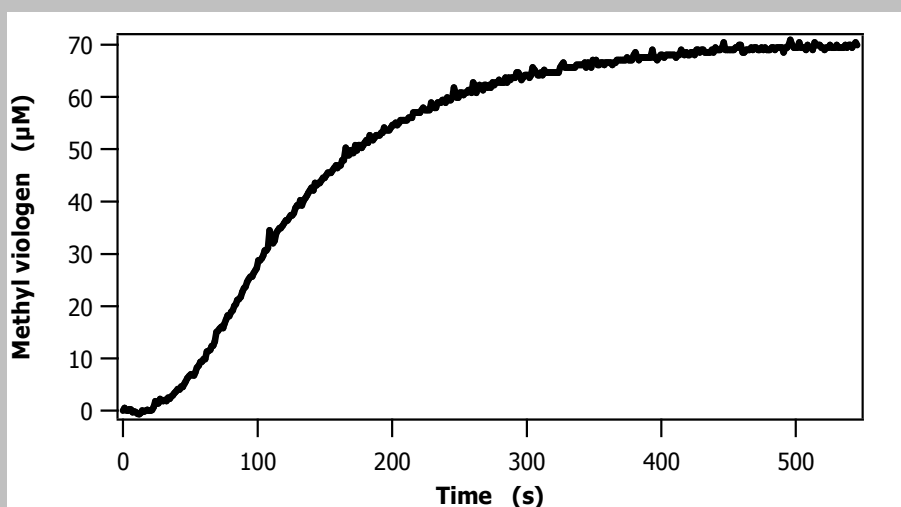


Figure 2. An activity trace for methyl viologen reduction by *D. vulgaris* Fe-hydrogenase. Activity was followed optically via the color of the forming semiquinone radical. Note the initial enzyme activation period over circa 90 s.

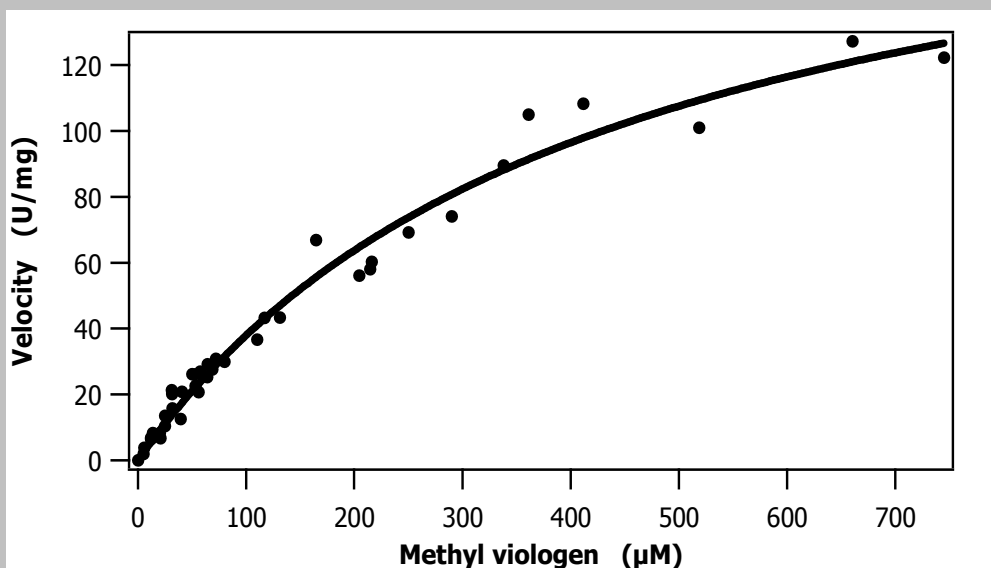


Figure 3. A plot of the velocity versus substrate concentration for the reduction of methyl viologen by activated *D. vulgaris* Fe-hydrogenase. The solid trace is a fit to the Michaelis-Menten equation with $K_M = 423 \mu\text{M}$ and $V_{\max} = 198 \text{ U/mg}$.

The K_M for methyl viologen is $0.42 \pm 0.4 \text{ mM}$ with a $V_{\max}$ of $198 \pm 12 \text{ U/mg}$.

The K_M can also be determined from a single activity trace, such as in Figure 2, by progress-curve analysis [10], i.e. by simulation of activity traces with a stepwise time-integrated rate equation. If this rate equation (in this case the Michaelis-Menten equation) properly describes enzyme activity, then the two methods must afford identical parameter values. Progress-curve analysis gives $K_M \approx 400 \mu\text{M}$ which is identical within experimental error to the value found in Figure 3.

K_M for hydrogen in the hydrogen oxidation reaction

When the hydrogen consumption activity at varying initial hydrogen concentrations is measured by polarographic monitoring of hydrogen, an enzyme activation phase is also observed (Figure 4A). Again, the rates of activated enzyme can be plotted versus the corresponding extrapolated substrate concentrations, and the data can be fitted to the Michaelis-Menten equation (Figure 5). The graph has a very shallow slope affording only an

estimate of the substrate concentration required for half maximal turnover $K_M \approx 2$ mM with a $V_{max} \approx 1800$ U/mg. Similar high numbers (i.e. 0.1-1 mM) have previously been reported as $K_{0.5}$ or K_M values for other Fe-hydrogenases [6, 11, 12], and they have been taken to indicate that the enzyme's prime physiological function is in the generation of hydrogen [13] although most physiological models propose a hydrogen oxidation function for the periplasmic *D. vulgaris* enzyme [14, 15].

Figure 4 A.

A set of activity traces for H_2 oxidation by *D. vulgaris* Fe-hydrogenase. The activity was followed polarographically on the H_2 disappearance. Note the initial enzyme activation period over ca. 90 s. Also note that for the activated enzyme any given H_2 concentration corresponds to a multitude of rates depending on the initial H_2 concentration.

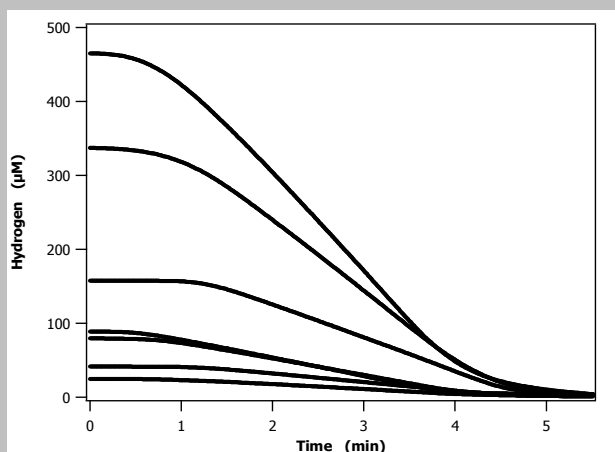
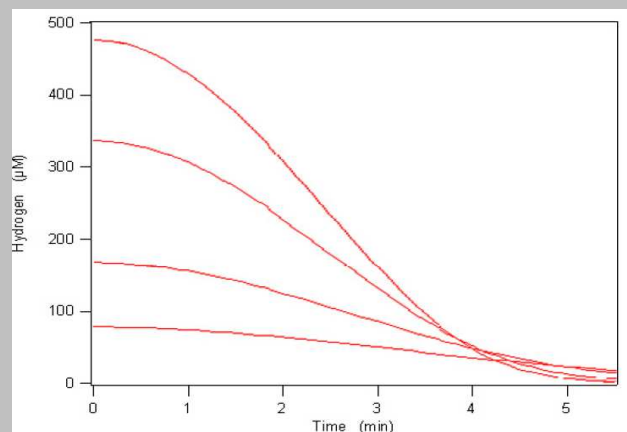


Figure 4B.

Simulation of the activity traces using the kinetic model described in the text and in Figure 8 (see Figure 8 for fitting k -values).



A possible way out of this apparent contradiction is suggested by progress-curve analysis of individual polarographic activity traces for hydrogen consumption [10]. An initial period of circa 90 s, in which the enzyme is activated, is followed by a phase of constant rate,

which eventually levels off when substrate concentration is reduced to a value comparable to the binding-affinity constant for the active center. Unexpectedly, this K_M -value of the hydrogen concentration (corresponding to a rate equal to half of the $V_{\max}$) ≈ 10 -20 μM . This number is circa two orders-of-magnitude less than the K_M -value estimated from the experiment in Figure 5.

When activity traces for different initial substrate concentrations are stacked in one graph, none of the traces overlap (Figure 4A). At any given hydrogen concentration there are different specific hydrogen consumption rates of activated enzyme apparently depending on the initial hydrogen concentration of the particular experiment.

How can the results of the progress-curve analysis in Figure 4A and the Michaelis-Menten fit to Figure 5 be so different, and what can we conclude from the observation that the traces in Figure 4A do not overlap? The kinetics must be drastically deviating from one-substrate Michaelis-Menten kinetics. However, the second substrate, methyl viologen, behaves normally. Therefore, we investigated the kinetics of the hydrogen activation more closely.

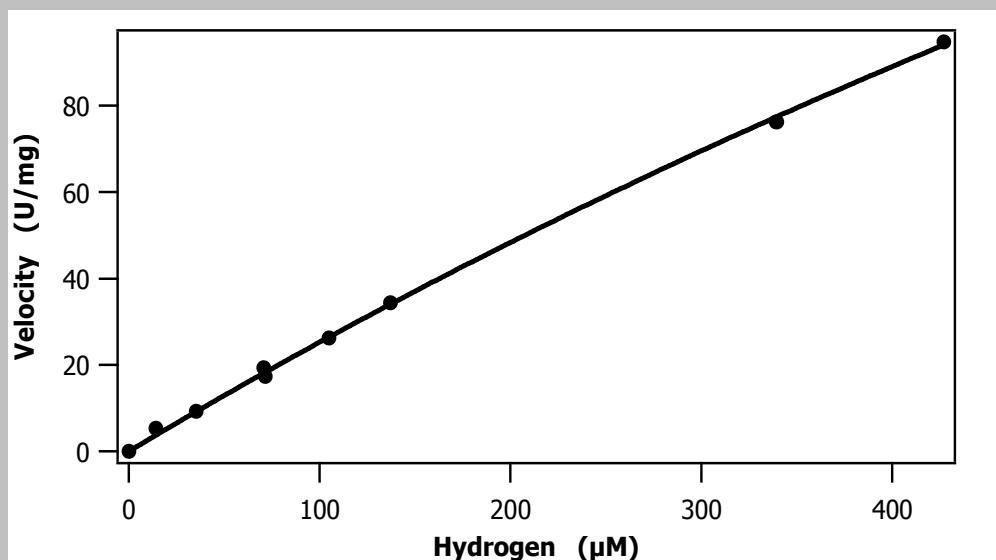


Figure 5. A plot of the velocity versus substrate concentration for the oxidation of molecular hydrogen by intermediately activated *D. vulgaris* Fe-hydrogenase. The solid trace is a fit to the Michaelis-Menten equation with $K_M = 2 \text{ mM}$ and $V_{\max} = 1800 \text{ U/mg}$.

Pre-activation with hydrogen affords fully active enzyme

When *D. vulgaris* Fe-hydrogenase is pre-incubated for 30 min. under 1 atm. of hydrogen overpressure the enzyme becomes fully active in the hydrogen oxidation assay: when the reaction is initiated by addition of enzyme, no activation phase is observed. The hydrogenase is immediately active and the activity is an order of magnitude higher than the maximal activity observed when the enzyme is activated in the course of the activity measurement. However, with the fully active enzyme there is no phase of constant rate: the rate of hydrogen consumption continuously decreases from time zero (cf. the traces in Figure 6).

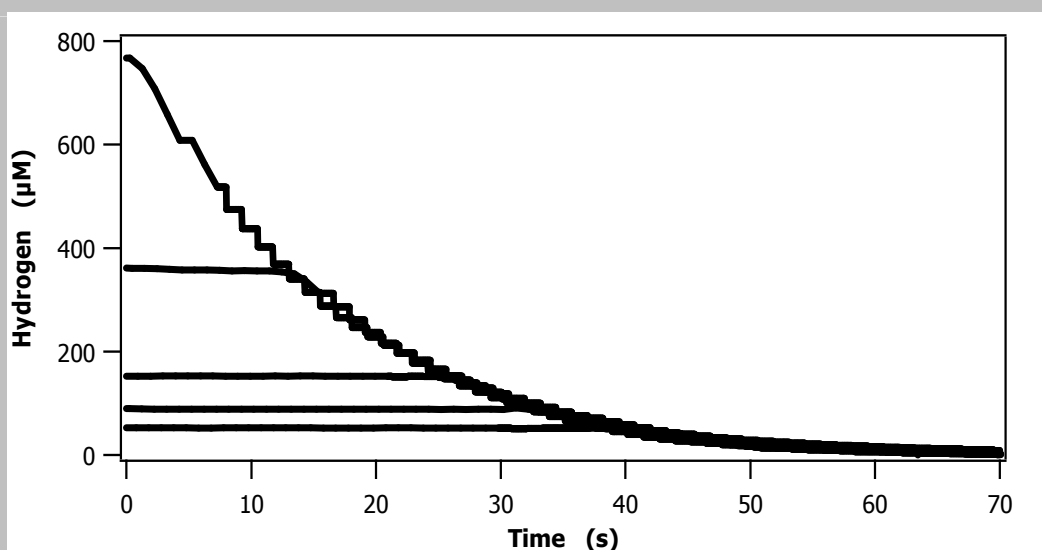


Figure 6. A set of activity traces for H_2 oxidation by fully active *D. vulgaris* Fe-hydrogenase. The activity was followed polarographically on the H_2 disappearance. The enzyme was activated by incubation under 1 atm of H_2 overpressure for 30 minutes. The traces with lower initial substrate concentration have been off-set on the time axis to show complete overlap of all traces for any given H_2 concentration. The step function in the data results from the interface setting to collect 10 averaged data per second.

The initial rates of the hydrogen consumption activity are plotted versus the hydrogen concentration in Figure 7. A fit of the data to the Michaelis-Menten equation gives $K_M \approx 1$ mM and $V_{\max} \approx 16000$ U/mg. When the different activity traces are stacked in one graph (Figure 6), and a time-shift is applied to correct for different hydrogen-concentration starting values, the traces are found to overlap, defining one unique specific hydrogen consumption activity at any given hydrogen concentration.

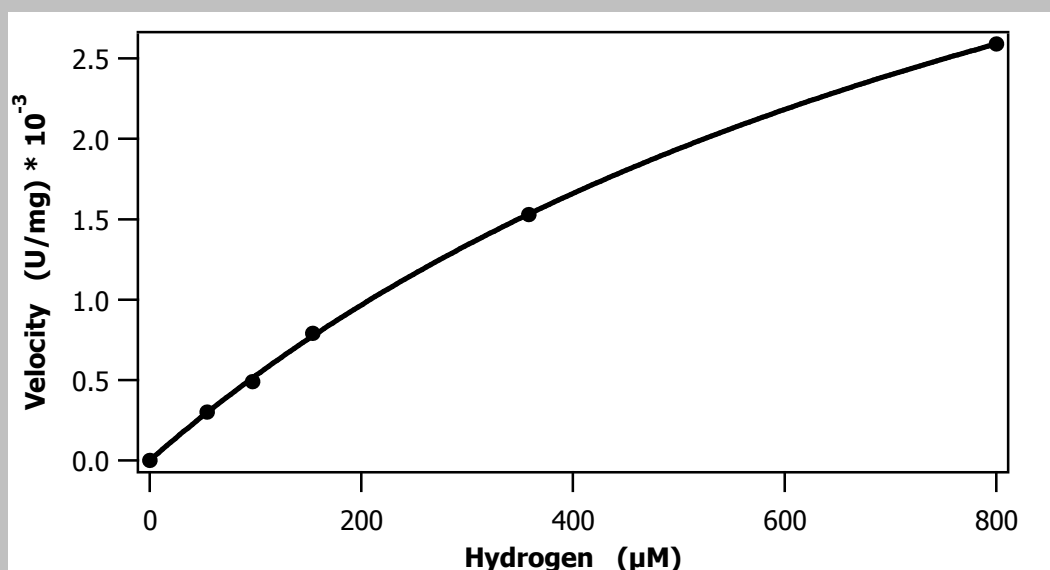


Figure 7. A plot of the initial velocity versus substrate concentration for the oxidation of molecular hydrogen by fully activated *D. vulgaris* Fe-hydrogenase. The solid trace is a fit to the Michaelis-Menten equation with $K_M = 1$ mM and $V_{\max} = 16000$ U/mg.

A kinetic model for hydrogen oxidation: activation and cooperativity

In hydrogen oxidation measurements *D. vulgaris* Fe-hydrogenase exhibits three different enzyme forms. A resting form, E_{rest} , (i.e. as isolated protein) is slowly (tens of seconds) activated under turnover conditions to active enzyme of relatively low activity, E_{inter} . Also, this active enzyme is very slowly (tens of minutes) further activated under hydrogen overpressure to E_{act} . Furthermore, active enzyme behaves distinctly non-Michaelis-Menten-

like as evidenced by two orders of magnitude difference in apparent K_M when determined from a Michaelis plot versus progress-curve analysis.

A minimal kinetic model that accommodates these boundary conditions has been developed and tested on the data showing non-Michaelis behavior in Figure 4. The mechanism is illustrated by the kinetic scheme presented in Figure 8, and it is described by the following differential equations:

$$dE_{\text{rest}}/dt = k_{-1} \cdot E_{\text{inter}} - k_1 \cdot S \cdot E_{\text{rest}} \quad \text{Equation 4}$$

$$dE_{\text{inter}}/dt = -k_{-1} \cdot E_{\text{inter}} + k_1 \cdot S \cdot E_{\text{rest}} + k_{-2} \cdot E_{\text{act}} - k_2 \cdot S \cdot E_{\text{act}} \\ + k_{-3} \cdot (ES)_{\text{inter}} - k_3 \cdot S \cdot E_{\text{inter}} + k_4 \cdot (ES)_{\text{inter}} \quad \text{Equation 5}$$

$$dE_{\text{act}}/dt = -k_{-2} \cdot E_{\text{act}} + k_2 \cdot S \cdot E_{\text{inter}} + k_{-5} \cdot (ES)_{\text{act}} - k_5 \cdot S \cdot E_{\text{act}} + k_6 \cdot (ES)_{\text{act}} \quad \text{Equation 6}$$

$$d(ES)_{\text{act}}/dt = -k_{-5} \cdot (ES)_{\text{act}} + k_5 \cdot S \cdot E_{\text{act}} - k_6 \cdot (ES)_{\text{act}} \quad \text{Equation 7}$$

$$d(ES)_{\text{inter}}/dt = -k_{-3} \cdot (ES)_{\text{inter}} + k_3 \cdot S \cdot E_{\text{inter}} - k_4 \cdot (ES)_{\text{inter}} \quad \text{Equation 8}$$

Since there are five unknowns we can eliminate one of these equations (e.g., Equation 5) by making use of the additional boundary condition for the known total enzyme concentration:

$$E_{\text{total}} = E_{\text{rest}} + E_{\text{inter}} + E_{\text{act}} + (ES)_{\text{inter}} + (ES)_{\text{act}} \quad \text{Equation 9}$$

Then, by solving Equations 4 and 6-9 for given k 's and initial substrate concentration the progress curve is stepwise generated by finding the substrate concentration after dt seconds:

$$dS/dt = -k_1 \cdot S \cdot E_{\text{rest}} - k_2 \cdot S \cdot E_{\text{inter}} - k_3 \cdot S \cdot E_{\text{inter}} - k_5 \cdot S \cdot E_{\text{act}} \\ + k_{-1} \cdot E_{\text{inter}} + k_{-2} \cdot E_{\text{act}} + k_{-5} \cdot (ES)_{\text{act}} + k_{-3} \cdot (ES)_{\text{inter}} \quad \text{Equation 10}$$

The biochemical interpretation of this scheme is as follows. The Fe-hydrogenase from *D. vulgaris* is isolated under aerobic conditions in a resting form [9]. The enzyme is activated in circa 90 s under turnover conditions, i.e. requiring the presence of hydrogen. The nature of the activation is not known but may perhaps be envisioned to involve the removal of an oxo

or peroxo ligand from de-activating molecular oxygen. The back-reaction is extremely slow as non-denaturing inactivation occurs during purification on a time scale of hours. For the kinetic analysis of activity traces (cf Figure 4) $k_{-1} \approx 0$ is thus a reasonable assumption. The active enzyme can now bind H_2 in two different ways (or both ways), one has mainly catalytic character, and the other has mainly regulatory character. The ‘catalytic’ binding site has a high affinity for H_2 (low K_D) but the resulting enzyme-substrate complex has a low turnover rate. The reaction course to product formation (the activity trace) exhibits a relatively long intermediate period of steady slope, but it has a very low initial rate. The ‘regulatory’ binding site has a low affinity for H_2 (high K_D) but the ternary enzyme-substrate-substrate complex has a high turnover rate. The reaction course for product formation shows no steady slope and has a high initial rate. The regulatory H_2 dissociates readily from the complex at lower dissolved hydrogen concentrations converting the fully active enzyme back to the intermediate form.

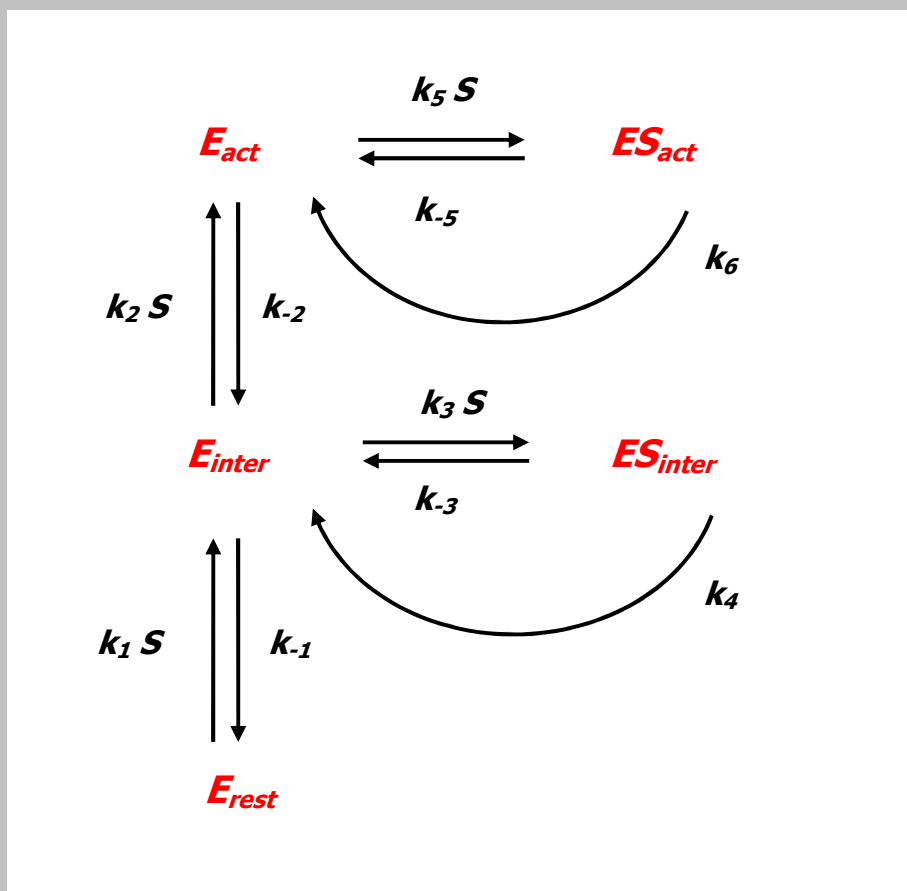


Figure 8. Proposed kinetic scheme for the intermediate activation and the full activation of *D. vulgaris* Fe-hydrogenase and its interaction with a catalytic and a regulatory H_2 molecule.

The rate constants used for the fit in Figure 4B are $k_1 = 1 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = 0 \text{ s}^{-1}$, $k_2 = 500 \text{ M}^{-1}\text{s}^{-1}$, $k_{-2} = 1 \text{ s}^{-1}$, $k_3 = 1 \cdot 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-3} = 100 \text{ s}^{-1}$, $k_4 = 1500 \text{ s}^{-1}$, $k_5 = 1 \cdot 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-5} = 100 \text{ s}^{-1}$, $k_6 = 8000 \text{ s}^{-1}$. Note that this fit is not unique but only serves to illustrate plausibility of the model.

Concluding Remarks

The proposal that two molecules of hydrogen can bind simultaneously to the active site of a hydrogenase raises a plethora of ramifying questions. What is the binding mode? Does, for example, one hydrogen bind as a metal bridging hydride and the other as a metal end-on hydrogen, or vice versa? Or is one hydrogen replaced from its original binding site by the other? Is the cooperativity of physiological advantage? Or is it perhaps simply a necessity dictated by chemical principles ruling the reactivity of the active site? Is this a general phenomenon for Fe-hydrogenases and perhaps also for NiFe-hydrogenases? How do predictions from theoretical chemistry, e.g., DFT calculations, change when allowance is made for binding of two hydrogen molecules? To what extent can reaction intermediates be followed spectroscopically? We do know that the resting *D. vulgaris* Fe-hydrogenase is diamagnetic while an oxidized, activated form of the enzyme exhibits unusual EPR ($g \approx 5$) possibly from an $S=2$ system [16]. Also, during reductive activation of the *D. vulgaris* enzyme an intermediate form of the active site is transiently observed in EPR ($g = 2.07, 1.96, 1.89$; cf [9] and Refs quoted therein). Would it be possible to resolve proton signals from two different substrate molecules in turnover intermediates by means of advanced spectroscopy, e.g., ENDOR or ESEEM (cf [17])? And what, finally, are the implications for projected technological applications of hydrogenase and for the synthesis and application of functional model compounds? These questions should be addressed in future hydrogenase research.

Acknowledgements

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Chapter 4

A Re-Investigation of the Steady-State Kinetics of the Soluble NiFe-Hydrogenase-I of Pyrococcus furiosus

Abstract

Pyrococcus furiosus has two types of NiFe-hydrogenases: a heterotetrameric soluble hydrogenase, and a multimeric transmembrane hydrogenase. Originally, the soluble hydrogenase was proposed to be “a new type of evolution hydrogenase”, because, in contrast to all then known NiFe-hydrogenases, hydrogen production activity at 80°C was found to be higher than the hydrogen consumption activity; NADPH was proposed to be the electron donor. Later it was found, that the membrane bound hydrogenase exhibits very high hydrogen production activity sufficient to explain cellular H₂ production levels, and this would seem to obliterate the need for a soluble hydrogen production activity and therefore leave the soluble hydrogenase without a function. Therefore the steady-state kinetics of the soluble hydrogenase were re-investigated. In contrast to previous reports a low $K_M \approx 20 \mu\text{M}$ for H₂ is found, which suggests a relatively high affinity for hydrogen. Also, the hydrogen consumption activity is an order of magnitude higher than the hydrogen production activity. These results appear to disqualify the soluble hydrogenase to function as a novel type of hydrogen production enzyme; they rather suggest that this enzyme is functionally similar to its NAD-reducing soluble multimeric NiFe-hydrogenase congener in *Ralstonia eutropha* with a $K_M = 37 \mu\text{M}$ for H₂. The soluble *P. furiosus* hydrogenase has $K_M \approx 37 \mu\text{M}$ for NADP⁺. We conclude that the soluble hydrogenase from *P. furiosus* is likely to function in the regeneration of NADPH thereby re-using the hydrogen produced by the membrane bound hydrogenase in proton respiration.

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Introduction

Hydrogenase catalyses the reversible conversion of molecular hydrogen into two protons and two electrons [1]. Although hydrogenases occur in a wide variety of monocellular anaerobes from each of the three domains of life, they all have a structural blueprint in common: The active site encompasses an organometallic cofactor based on a sulfur-bridged (Fe, Fe) or (Fe, Ni) pair of metal ions with CO, CN⁻ ligands on the Fe, with hydrophobic and hydrophilic channels to the protein's surface for transport of H₂ and of protons, and with a [4Fe-4S] cubane for electron transfer. In Fe-hydrogenases the cubane is integral part of the active site via a bridging cysteine sulfur; in Ni-hydrogenases the cubane is in a separate subunit, however, it is spatially close to the dinuclear center, and it is thus designed 'proximal' cluster.

Pyrococcus furiosus is a strict anaerobe and a hyperthermophilic marine archaeon that grows optimally at a temperature of 100 °C by saccharolytic fermentation or by S⁰-dependent peptidolytic fermentation [2]. *P. furiosus* has two types of hydrogenases: a coenzyme-dependent heterotetrameric soluble enzyme and a multimeric (14 subunits) transmembrane enzyme. Both hydrogenases are encoded polycistronically. A duplication of the operon for the soluble enzyme encodes a low-activity paralog, designated soluble hydrogenase-II that is expressed significantly during growth on maltose [3]. There is also a duplication of the operon for the membrane-bound hydrogenase, and transcription of this operon (13 ORFs) has been established [4]. All four *P. furiosus* hydrogenases are of the NiFe-type, however, the putative second membrane-bound enzyme has a structural gene in which the codons for the bridging Cys ligands to the dinuclear NiFe center are replaced with codons for a glutamate and an aspartate, which would be unprecedented substitutions [5].

The physiological role of the soluble hydrogenase(s) in *P. furiosus* is not clear; the literature documents several subsequent re-assignments of putative function. Originally, the enzyme was called a new type of evolution hydrogenase because, in contrast to all known NiFe-hydrogenases, H₂ evolution activity at 80 °C was found to be higher, by approximately four-fold, than H₂ oxidation activity [6]. Ferredoxin was proposed to be the natural electron donor [6]. It was subsequently found that the enzyme also has S⁰ reduction activity, and could therefore be a bifunctional "sulfhydrogenase" [7]. Then, it was found that the direct reaction

with ferredoxin is artifactual, and the natural electron donor was proposed to be NADPH, in its turn formed by the action of the enzyme ferredoxin: NADP⁺ oxidoreductase [3, 8]. Reported midpoint potential values for the iron-sulfur clusters are relatively high for H₂ production [9, 10]. Later, it was found that *P. furiosus* membranes contain a membrane-bound hydrogenase with high H₂ production activity [11], and this would seem to obliterate the need for a – lower – soluble H₂ production activity, and, therefore, would leave the soluble enzyme without an identified function. In another study on the membrane-bound hydrogenase the ferredoxin was identified as the redox partner [5]. It was also determined that the hydrogenase activity was sensitive to the proton-translocation inhibitor dicyclohexylcarbodiimide, and the activity of the soluble hydrogenase was insufficient to explain H₂ production in vivo [5]. This led to the proposal of proton respiration coupled to energy transduction in a transmembrane hydrogenase complex [5], which was later verified by the observation of ATP production coupled to the production of H₂ in membrane vesicles [12]. The soluble hydrogenase was proposed to function as a safety valve for the disposal of excess reducing equivalents and/or as a conversion system that would re-use the waste product of respiration, H₂, for the production of NADPH for anabolic metabolism [5]. The latter assignment would make the soluble hydrogenase a hyperthermophilic equivalent of conventional NiFe-hydrogenases, and this would appear to be inconsistent with the earlier reports on high production activity [6] and on a relatively high K_M value for H₂ in the oxidation reaction [3]. We have therefore re-investigated the steady-state kinetics of the soluble hydrogenase.

Material and Methods

Growth of *Pyrococcus furiosus*

P. furiosus (DSM 3638) was routinely grown at 90°C in a 140 liter fermentor with starch as the carbon source as described previously [5].

Protein analysis

The protein concentration was determined with the Bichinchoninic acid (BCA) assay [13]. The absorbance was determined at 562 nm with bovine serum albumine as standard.

Enzyme activity assays

Methyl viologen reduction activity was measured optically in a glass fiber CCD array spectrophotometer (Avantes AVS-S2000), by the increase of absorption at 600 nm using $\epsilon_{600} = 9.7 \text{ mM}^{-1}\text{cm}^{-1}$ (or at 500 nm for high viologen concentrations; $\epsilon_{500} = 2.0 \text{ mM}^{-1}\text{cm}^{-1}$) at 80°C under anaerobic conditions in a 1 ml rubber-stoppered cuvette. The cuvette was filled with a given concentration of methyl viologen in 100 mM Epps, pH 8.0, and saturated with hydrogen by bubbling. The reaction was initiated by addition of enzyme. One unit of activity is defined as two μmol of methyl viologen reduced to the semiquinone radical per minute. In a similar protocol, NAD(P)^+ reduction activity was measured by the increase of absorption at 340 nm ($\epsilon_{340} = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$). One unit of activity is one μmol of NAD(P)H formed per minute.

Hydrogen consumption activity was measured amperometrically under anaerobic conditions with a Clark-type electrode (YSI model 5331 from Yellow Springs, USA) polarized at 600 mV versus Ag/AgCl in a 2 ml water-jacketed cell. The temperature was held at 80°C by circulating water. The dual electrode was interfaced to a PC via a National Instruments 16 bits ATD converter, and current data were collected with a Lab-View program at 0.1 s intervals. The assay mixture was 1 mM methyl viologen in 1 ml 100 mM Epps, pH 8.0, and variable concentration of hydrogen obtained by dilution with a buffer solution saturated with hydrogen. The solubility of hydrogen at 80°C is 745 μM [14]. The reaction was initiated by addition of enzyme. One unit of enzyme activity is defined as one μmol of hydrogen oxidized per minute.

Results and Discussion

In different physiological contexts hydrogenases function either in the consumption or in the production of molecular hydrogen. According to a long-standing paradigm NiFe-hydrogenases frequently are H_2 -uptake enzymes with an apparent high affinity for H_2 as reflected in a Michaelis constant for H_2 in the micromolar range; contrarily, Fe-hydrogenases are thought to function as H_2 -production catalysts with a high $K_{\text{M}}(\text{H}_2)$ in the hundreds of

micromolar range [1] [Chapter 2]. A second function-discerning criterion is the ratio of uptake activity over production activity of purified enzymes, which is typically 100-1000 for NiFe-hydrogenases and circa 10 for Fe-hydrogenases [1]. It came, therefore, as a complete surprise when the first description of a soluble NiFe-hydrogenase from *P. furiosus* reported an uptake over production activity ratio at 80 °C of ≈ 0.25 [6]. The suggestion that this enzyme could be the first representative of a novel production-type NiFe-hydrogenase [6, 8] was later corroborated by the finding of a $K_M(\text{H}_2)$ value of 140 μM [3], which is one-two orders-of-magnitude greater than typical values for mesophilic NiFe-hydrogenases [Chapter 2]. Recently, however, we have approached the steady-state kinetics of hydrogenases from a thus far unexplored angle, namely by means of progress-curve analysis [Chapter 2], and our results for the soluble *P. furiosus* enzyme suggested a much higher affinity for hydrogen as evident from an apparent value of $K_{0.5}(\text{H}_2) \approx 5 \mu\text{M}$ that is an order of magnitude less than the previously reported K_M value.

To resolve this apparent inconsistency we have now also carried out a Michaelis-Menten analysis of this reaction, and the results are summarized in Figure 1. The experiment was done under conditions (80 °C; 100 mM Epps buffer, pH 8) identical to those employed in previous work by Ma et al. [3] except that the substrate H_2 , for which the K_M was to be determined, was directly monitored amperometrically. For the second substrate we used not only 1 mM methyl viologen, but also 1 mM benzyl viologen because its $E_{m,7}$ is less negative than that of the H_2/H^+ couple, and because in early work unusual behaviour of methyl viologen was reported for this reaction [6] (see also below). No activation was required for the anaerobically kept enzyme. Shortly ($< 5 \text{ s}$) after enzyme injection in the Clark cell a steady rate of H_2 disappearance was reached and this was taken as the ‘initial’ velocity values for the abscissa of Figure 2. The data can be seen to be well fit by simple Michaelis-Menten kinetics with a $V_{\max}$ of circa 900 U/mg and a $K_M(\text{H}_2)$ of 18 μM for the reaction with benzyl viologen as the electron acceptor, and a $V_{\max}$ of circa 942 U/mg and a $K_M(\text{H}_2)$ of 20 μM for the reaction with methyl viologen as the electron acceptor. The K_M values are of the same order as the $K_{0.5}(\text{H}_2) \approx 5 \mu\text{M}$ previously estimated in progress-curve analysis with methyl viologen as the acceptor (Chapter 2)

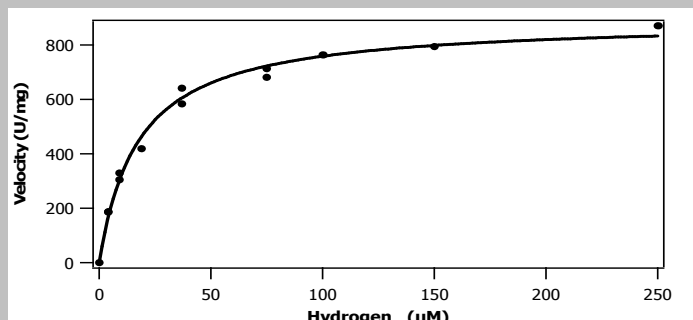


Figure 1A. Michaelis-Menten curve of H₂ for the reaction with benzyl viologen
 $K_M = 17.5 \pm 1.5 \mu\text{M}$;
 $V_{\text{max}} = 891 \pm 21 \text{ U/mg}$

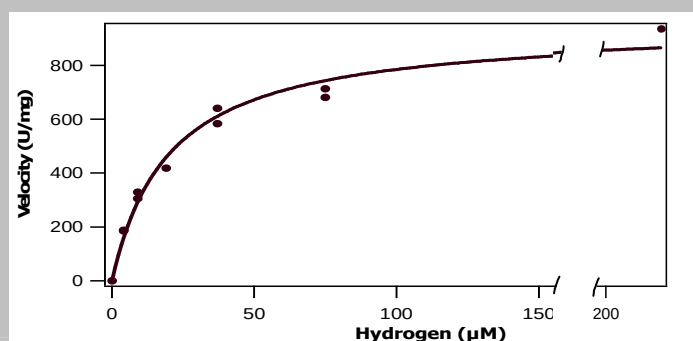


Figure 1B. Michaelis-Menten curve of H₂ for the reaction with methyl viologen
 $K_M = 20.1 \pm 2.8 \mu\text{M}$;
 $V_{\text{max}} = 942 \pm 42.9 \text{ U/mg}$

As an independent check the second substrate, oxidized viologen, was varied while the first substrate, H₂, was kept constant at saturating concentration. Previously, Ma et al have reported a 25-fold difference in the two V_{max} values for the reaction of H₂ with methyl viologen, where the higher value refers to the experiment in which the viologen was varied at constant H₂ concentration [3, 8] Ma et al. report an extremely high $K_M(\text{MV}_{\text{ox}})$ of 5 mM, which would partially explain the large difference in the two V_{max} values. It would also imply that the standard assay of H₂ oxidation activity with 1 mM methyl viologen is a rather suboptimal one, namely at 1/6 of V_{max} . Our results do not reproduce these previous reports. The solubility of hydrogen in water at 80°C under 1 atm of H₂ is 745 μM [14], which is more than an order of magnitude above the here reported K_M values for H₂.

The variable substrate (viologen) was detected directly by its optical absorption, and the resulting initial velocities again fit simple Michaelis-Menten kinetics (Figure 2) for a V_{max} of circa 800 U/mg and a $K_M(\text{viologen}_{\text{ox}}) = 0.19 \text{ mM}$ in the reaction with benzyl viologen, and a V_{max} of circa 1400 U/mg and a $K_M(\text{viologen}_{\text{ox}}) = 0.13 \text{ mM}$ in the reaction with methyl viologen. The K_M values imply that the H₂-variation experiments were done with a viologen concentration (1 mM) well above its K_M and, therefore, that the two experiments (variation of

H₂ versus variation of viologen) should afford similar values for $V_{\max}$, which is indeed approximately the case.

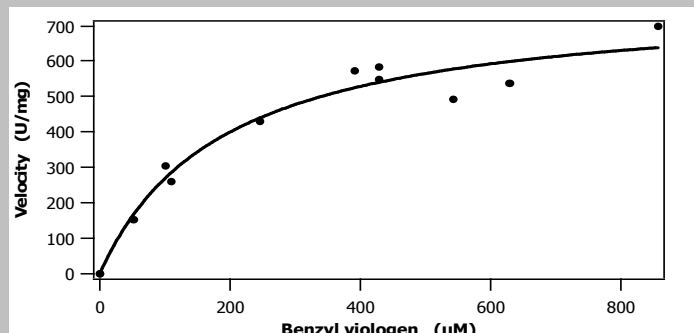


Figure 2A. Michaelis-Menten curve of benzyl viologen
 $K_M = 190 \pm 46 \mu\text{M}$;
 $V_{\max} = 778 \pm 62 \text{ U/mg}$

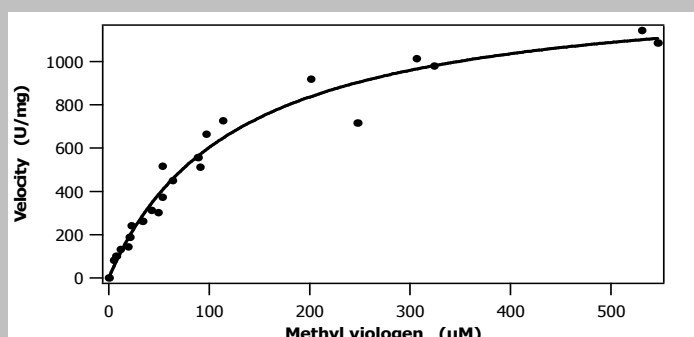


Figure 2B. Michaelis-Menten curve of methyl viologen;
 $K_M = 126 \pm 15 \mu\text{M}$;
 $V_{\max} = 1361 \pm 68 \text{ U/mg}$

Originally, for the soluble hydrogenase hydrogen oxidation activity at 80 °C was found to be lower, by approximately four-fold, than hydrogen production activity [6]. However, we have later found that the production activity at 80 °C was approximately the same as the consumption activity at 60 °C, suggesting that the ratio of consumption activity over production activity at the same temperature is significantly greater than unity [15]. We have now re-determined these activities both at 80 °C: the hydrogen consumption activity is circa eight times higher than the hydrogen production activity.

Finally, NADPH has been proposed by Ma et al to be the physiological electron donor for the soluble hydrogenase in H₂ production [8], but this has been challenged by Silva et al who proposed that NADP⁺ be the electron acceptor for the enzyme in H₂ consumption [5]. In the latter case one would expect the $K_M(\text{NADP}^+)$ to be in the micromolar range, and a value of 40 μM was indeed reported by Ma et al [3, 8] We have repeated this experiment and find a similar $K_M(\text{NADP}^+) = 37 \mu\text{M}$ (Figure 3).

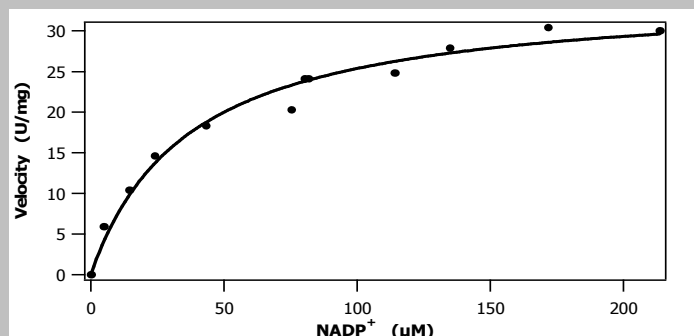


Figure 3. Michaelis-Menten curve of NADP⁺;

$$K_M = 37 \pm 2 \mu\text{M};$$

$$V_{\text{max}} = 34 \pm 5 \text{ U/mg}$$

In conclusion, for the soluble hydrogenase-I of *P. furiosus* we find a $K_M(\text{H}_2)$ of circa 20 μM , which is within the range of values reported for other NiFe-hydrogenases and, particularly, similar to the value of 37 μM reported for the soluble, NAD⁺ reducing, multi-subunit hydrogenase from *Ralstonia eutropha* [16]. Also, the K_M values of 0.1-0.2 mM for the oxidized viologens are not at all unusual. Furthermore, in our hands the hydrogen consumption activity is an order of magnitude greater than the hydrogen production activity. These observations taken together appear to disqualify the soluble *P. furiosus* enzyme to function as a novel type of production enzyme [3, 6, 8], and they suggest to support the proposal that this enzyme is kinetically similar to its NiFe-hydrogenase congeners in other microorganisms. We conclude that the soluble enzyme is likely to function in the regeneration of NADPH at the expense of H₂ produced by the membrane-bound hydrogenase in proton respiration [16].

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Chapter 4

Steady-State Kinetics of the Soluble NiFe-Hydrogenase

Chapter 5

The Intramolecular Electron Transfer Chain of the Soluble, NADPH-dependent Hydrogenase-I of Pyrococcus furiosus

Abstract

The heterotetrametric, soluble NiFe-hydrogenase-I of *Pyrococcus furiosus* contains a complex chain of iron-sulfur prosthetic groups for electron transfer between the H₂-activating NiFe site and the coenzyme-activating flavin. Previous EPR studies have identified only two Fe-S signals in reduced enzyme. In contrast to previous reports the H₂ production activity is found to be sensitive to carbon monoxide with 99% inhibition in CO saturated buffer at 80 °C. Incubation with dithionite under CO at ambient temperature essentially abolished H₂ production and affords stable, maximally reduced enzyme, whose EPR analysis reveals three, possibly four, different Fe-S components. In addition to two subspectra previously assigned to a [2Fe-2S]⁺ and a high-potential [4Fe-4S]⁺ cluster, there is a third, very anisotropic subspectrum reminiscent of the atypical spectrum of the Fe/S II center in xanthine dehydrogenase. Sequence analysis reveals no homology to this enzyme, but suggests the presence of one [2Fe-2S], five [4Fe-4S], and two unprecedented Fe/S binding motifs. The EPR silence of approximately half of the Fe/S clusters under optimized reducing conditions makes this hydrogenase another member of the group of multiheteromeric iron-sulfur flavin enzymes with Fe/S clusters of elusive redox properties.

Introduction

Although nature's catalyst for the activation of molecular hydrogen, hydrogenase, is widely distributed amongst anaerobic microorganisms, its active center is always constructed following a single general design: a cofactor, based on a sulfur-bridged (Fe,Fe) or (Fe,Ni) pair of metal ions with CO, CN⁻ ligands on the Fe, close to a [4Fe-4S] cubane for electron transfer [1, 2]. In contrast to this unifying topology electron transfer beyond the first cubane is variable to a considerable degree in structure, function, and compartmentalization, with system complexity ranging from a soluble single subunit hydrogenase in direct electron exchange with a soluble redox partner [3], to a hydrogenase that is part of a multi-subunit, multi redox group trans-membrane complex with proton pumping capacity [4, 5, 6]. A small group of intermediate complexity is formed by the coenzyme-dependent, soluble, multi-subunit hydrogenases, for which a prototypical example is the extensively studied *Ralstonia eutropha* enzyme [7]. These systems have electron transfer chains consisting of more than half a dozen redox centers, which are all contained in a single multimeric molecule.

The object of the present study, *Pyrococcus furiosus* soluble NiFe-hydrogenase-I, is also a member of this family. Based on physiological and kinetic studies we have proposed the enzyme to function in the reductive regeneration of coenzyme NADP⁺ with hydrogen as the reductant [8, 9]. The $\alpha\beta\gamma\delta$ -heterotetrameric protein has the NiFe dinuclear site for H₂ activation in the α -subunit, and it has a flavin, FAD, in the γ -subunit for coenzyme redox chemistry. The β - and δ -subunit plus part of the γ -subunit carry binding motifs for at least six iron-sulfur clusters presumably for electron transfer between the two active sites of the complex [10, 8]. Surprisingly, EPR spectroscopic studies have thus far identified a total of only three signals that can be ascribed to two or perhaps three different FeS clusters [11, 12, 8].

Due to the omnipresence of the substrate H⁺, chemical reduction and enzymic turnover of active hydrogenase cannot be separated, and the reduced state of FeS clusters may be difficult or impossible to obtain unless the enzyme's hydrogen production activity can be fully blocked by reversible inhibition. Carbon monoxide is a common reversible inhibitor of many NiFe-hydrogenases, however, the soluble hydrogenase-I of *P. furiosus* has been reported to be insensitive to CO [13, 14]. We have re-investigated this property, and we find

that the production of molecular hydrogen is in fact nearly completely inhibited under a CO atmosphere. The CO-inhibition is subsequently used to produce enzyme in which a maximum number of prosthetic groups is reduced with sodium dithionite. EPR analysis of the reduced enzyme affords identification of some of the redox centers that form the complex intramolecular electron transfer chain of this system.

Material and Methods

P. furiosus (DSM 3638) was grown and its soluble hydrogenase-I was isolated and purified as previously described [Chapter 4 of this thesis]. The concentration of purified enzyme was based on the nickel content as determined with a Hitachi 180-80 polarized Zeeman atomic absorption spectrophotometer using the graphite atomization method.

Hydrogen production activity was measured amperometrically under anaerobic conditions with a Clark-type electrode (YSI model 5331, Yellow Springs, USA) polarized at 600 mV versus Ag/AgCl in a 2 ml water-jacketed cell. The temperature was held at 80°C by circulating water. The dual electrode was interfaced to a PC via a National Instruments 16 bits ATD converter, and converted current data were collected with a LabView program at 1 s intervals. The assay mixture was 1 mM reduced methyl viologen in 2 ml 100 mM Epps, pH 8.0. A variable concentration of CO was added by injection of a specific volume of CO saturated Epps buffer solution. The solubility of CO at 80°C is 691 μM [15]. The reaction was initiated by addition of enzyme. One unit of enzyme activity is defined as one μmol of molecular hydrogen produced per minute.

EPR spectra were taken with a Bruker ER200D spectrometer [16]. The multi-component spectra were simulated assuming non-interacting species subject to *g*-strain [17, 18] and following the protocol recently described for multiple valence isomers in EPR spectra of HiPIP proteins [19].

Results and Discussion

CO inhibition

Complete insensitivity to CO up to 320 μM both in the H_2 uptake and H_2 production activity has been reported for *P. furiosus* soluble hydrogenase [13, 14]. Contrarily, we find that the production activity at 80 °C with reduced methyl viologen as the electron donor exhibits, in fact, significant sensitivity to CO with circa 82% inhibition at 320 μM and a maximum inhibition of $\geq 99\%$ in CO saturated buffer (i.e. 691 μM) as shown in Figure 1. At 20 °C the uninhibited H_2 production activity has dropped to a fraction of its value at 80 °C, and the activity in CO saturated solution is essentially zero. This allows for the stable reduction (i.e. no H_2 formation from solution protons) of the enzyme with a variety of reductants.

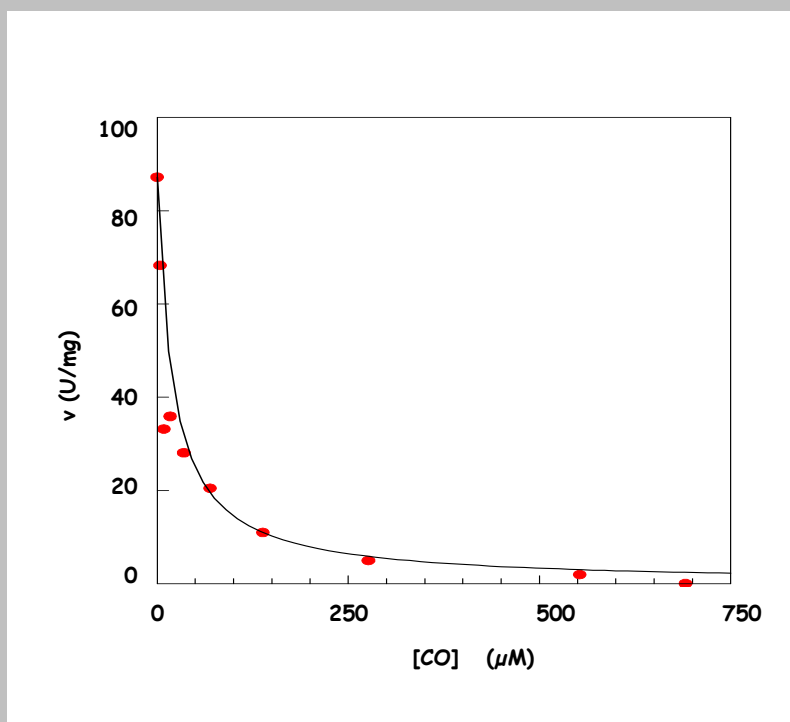


Figure 1. CO inhibition of H_2 production activity by *P. furiosus* hydrogenase-I at 80 °C. The solid line is a fit to the function $v = v_{\text{max}} / (1 + \gamma[\text{CO}])$ with $\gamma = 0.05$

EPR quantification of reduced enzyme

Incubation at pH 8.0 under 1 atm of H_2 ($E_{m,8.0} = -480$ mV) at 80 °C followed by freezing in liquid nitrogen affords an EPR spectrum of only weak intensity that integrates to less than 0.3 $S = 1/2$ spins per nickel atom as a measure of enzyme concentration (Figure 2., trace A). This indicates that quantitative reduction of the hydrogenase with H_2 is impossible because of considerable back-flow of electrons due to H^+ reduction activity. Therefore, the enzyme was incubated under 1 atm of CO at ambient temperature and subsequently chemically reduced with NADPH ($E_{m,8.0} = -350$ mV), sodium dithionite ($E_{m,8.0} \approx -0.56$ V at 10 mM [20]), and titanium(III) citrate ($E_{m,8} \approx -560$ mV [21]).

The latter has the disadvantage that the unreacted reductant has a strong EPR signal (Ti^{III} is $3d^1$, $S = 1/2$) which almost completely overlaps with typical $S = 1/2$ spectra of Fe-S clusters [22]. It has been used here to check for possible high-spin Fe-S signals at low magnetic fields. No such signals were detected either with $Ti(III)$ citrate plus CO or with dithionite plus CO (not shown).

Light reduction of the system deazaflavin / methyl viologen / EDTA is commonly used in biology to afford an even stronger reducing environment; however, this is not an option for the present case because the CO coordination is light sensitive.

Reduction with NADPH under CO affords the spectrum of trace B in Figure 2. The total integrated intensity of this spectrum gives a spin count of circa 1.3. Reduction with dithionite in the presence of CO gives the spectrum of Figure 2, trace C: the spin count has now increased to 2.5. The difficulty of determining the limits of spectral integration associated with the tailing wings of the spectrum cause a significant uncertainty in the spin count, which by extensive variation of limits and baseline choice was estimated to be approximately ± 0.5 spins.

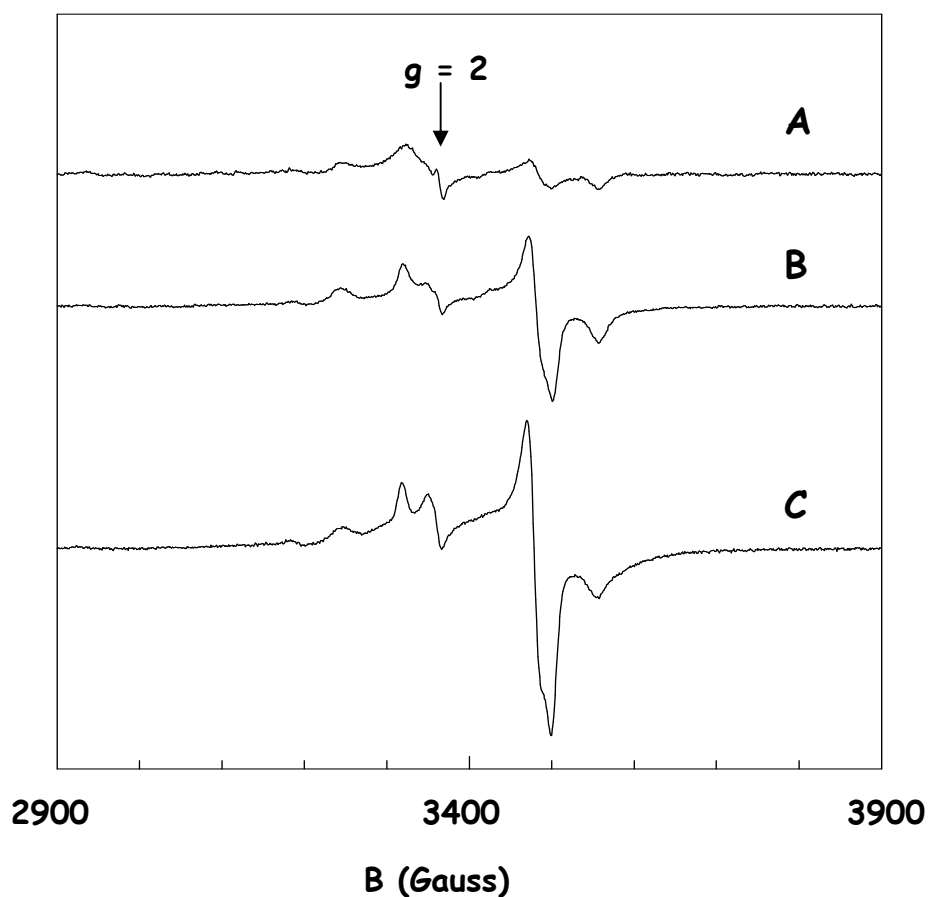


Figure 2. EPR spectra of *P. furiosus* hydrogenase-I reduced with 1 atm H_2 (trace A), with 10 mM NADPH in the presence of 1 atm CO (trace B), and with 10 mM sodium dithionite in the presence of 1 atm CO (trace C). EPR conditions: microwave frequency, 9.41 GHz; microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 8 Gauss, temperature, 16 K.

EPR analysis of inhibited, reduced enzyme

Sequence analysis (see below) suggests the presence of up to eight Fe/S centers. EPR of the maximally reduced enzyme exhibits a complex pattern of overlapping $S = 1/2$ systems. This is not an uncommon situation for iron-sulfur proteins, although the complexity of the present spectrum is rather pronounced, and full resolution of all individual components is not a realistic goal. For this type of problem we have recently worked out an approximating deconvolution scheme in the analysis of multiple valence isomers in high-potential iron-sulfur proteins (HiPIPs). The method is based on the assumptions that; (i) the shapes of all low-spin Fe/S X-band spectra are dominated by g -strain; (ii) the highest g -values (at lowest fields), g_z 's, are resolved; (iii) theory limits the possible g -values. For HiPIPs, e.g., the latter condition reads: all g -values are greater than the free electron value, $g_e = 2.0023$. A further restriction of this condition may come from extensive experimental observation: all g -values from HiPIPs are less than an approximate upper limit of $g_{\max} \approx 2.14$. Since in the present case no significant EPR intensity is observed for the oxidized enzyme, we only have Fe/S clusters of the ferredoxin type, i.e. $[4\text{Fe-4S}]^{1+}$ or $[2\text{Fe-2S}]^{1+}$, which means that the theoretical g -value limitation becomes: $g_z > g_e$, $g_y \leq g_e$, and $g_x < g_e$, with a further limitation based on many experimental observations that $2.14 \geq g > 1.7$. Valence isomers (cf in HiPIPs) are substoichiometric by definition; however, they have not been reported to be observable from ferredoxin-type clusters. For our present analysis this means that we have an additional boundary condition: (iv) each individual $S = 1/2$ spectrum of a fully reduced species should, under non-saturating microwave conditions, afford an approximate stoichiometric spin count.

The spectrum of fully CO-inhibited, dithionite-reduced hydrogenase is now analyzed through building up an overall simulation by subsequent addition of an increasing number of individual $S = 1/2$ components subject to the restrictions outlined above, until all discernable g_z peaks are covered. The result is given in Figure 3 and Table 1.

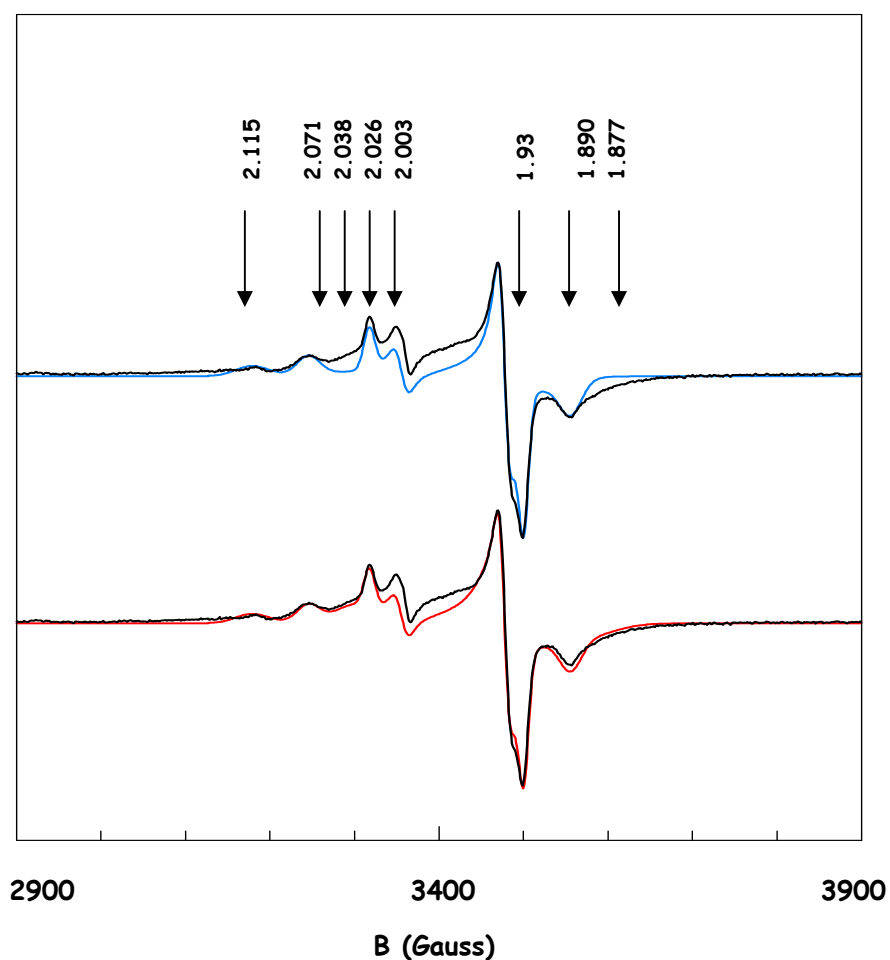


Figure 3. Multi-component deconvolution of the EPR spectrum of *P. furiosus* hydrogenase-I reduced with 10 mM dithionite in the presence of 1 atm CO. The fit parameters are in Table 1. The upper trace is a fit using the first three entries of Table 1; the lower trace is a fit that uses all four entries of Table 1. Inclusion of the fourth component may not be significant as it may simply mimic broadening by dipolar interaction between clusters.

	g_z	g_y	g_x	Δ_{zz}	Δ_{yy}	Δ_{xx}	ratio
Center-1	2.071	1.932	1.89	0.010	0.006	0.008	1
Center-2	2.026	1.933	1.92	0.0047	0.0036	0.0036	0.7
Center-3	2.115	2.003	1.9	0.015	0.005	0.02	0.7
Center-4^{a)}	2.038	1.935	1.977	0.015	0.01	0.02	1.0

^{a)} this component may be a fitting artifact; see the text.

Table 1. EPR simulation parameters of CO-inhibited dithionite-reduced *P. furiosus* hydrogenase-1.

Reliable analysis of multi-component EPR requires low-noise spectra, therefore, the fitting has been done on a spectrum taken at a partially saturating microwave intensity (Figure 3). The extent of saturation has been determined with power plots as illustrated in Figure 4. This approach assumes that individual spectral shapes do not significantly distort under mildly saturating conditions. Partial saturation experiments may also provide independent information on how to deconvolute multi-component spectra. In the present case two discernible saturation patterns were found (Figure 4), with $P_{0.5} = -11$ dB or > 0 dB, respectively (with a nominal output power of 200 mW at 0 dB attenuation), and each individual component behaves according to one of these two patterns. This observation sets an extra boundary condition: easily saturable features cannot be combined with not-easily saturable features into individual spectra. Furthermore, the derivative-shaped feature close to the free electron value is found to saturate in concert with the $P_{0.5} = -11$ dB components, and this excludes the assignment of this feature to a radical (which would saturate much more readily at this temperature).

The overall analysis shows that maximally 3-4 individual $S = 1/2$ systems can be discerned, and that each component has theoretically and experimentally (by comparison) acceptable g -values. Only three individual g_z lines are discernible (Figure 3), two of which correspond to previously identified spectra, namely a $[2\text{Fe-2S}]^{1+}$ cluster ($g_z = 2.026$, $P_{0.5} = -11$ dB) and a putative $[4\text{Fe-4S}]^{1+}$ cluster ($g_z = 2.071$, $P_{0.5} > 0$ dB) with unusually high reduction potential [12]. The third spectrum ($g_z = 2.115$, $P_{0.5} = -11$ dB) has not been previously recognized, although a “broad line around $g = 2.14$ ” was already reported by Bryant et al.

[11]. The spectrum is rather similar to that of the $[2\text{Fe-2S}]^{1+}$ cluster “Fe/S II” in xanthine dehydrogenase/oxidase which has an unusual set of g -values [23], but a not so unusual $\text{Cx}_4\text{Cx}_2\text{Cx}_{22}\text{C}$ binding motif [24]. The fourth spectrum ($g_z = 2.038$, $P_{0.5} > 0$ dB) is poorly resolved and might even be a “ghost” spectrum that mimics dipolar broadening.

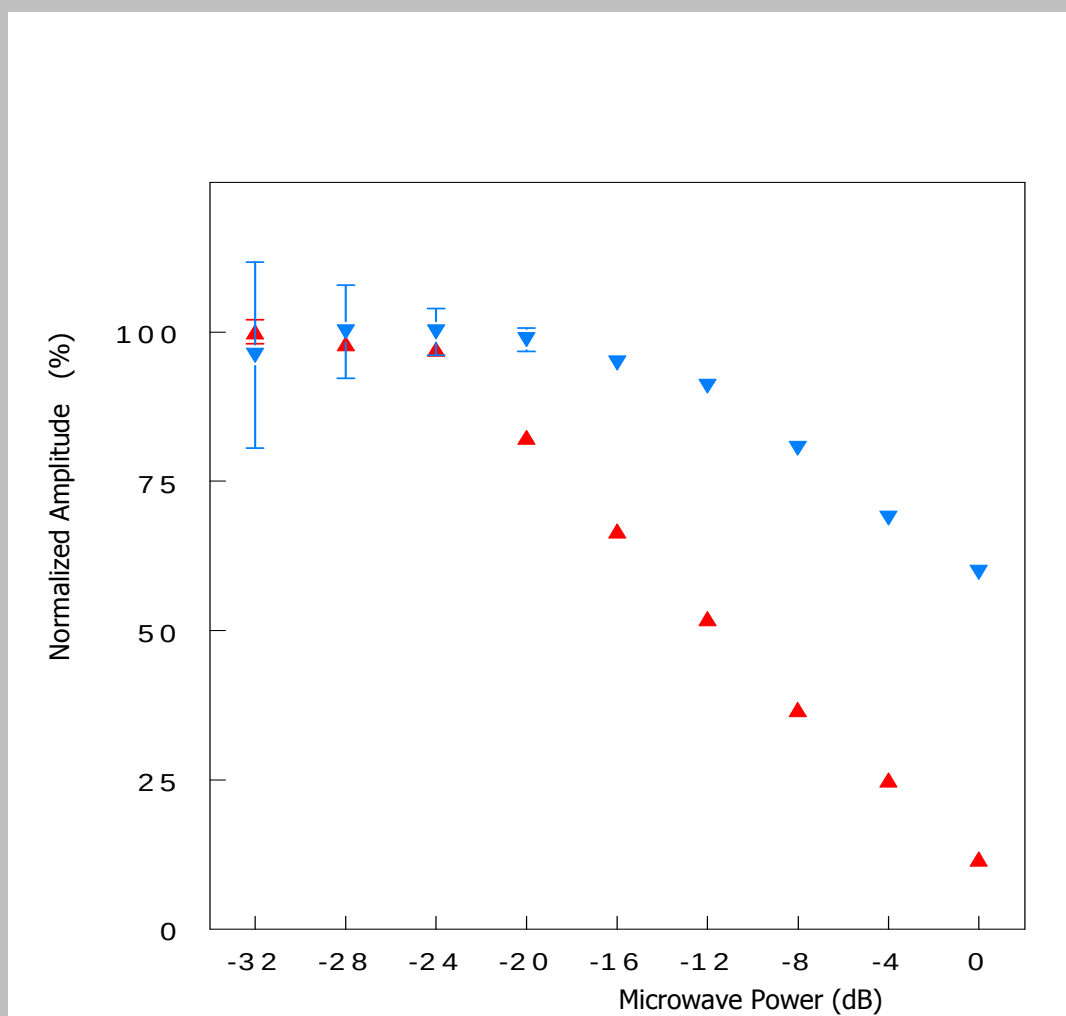


Figure 4. Saturation characteristics of the iron-sulfur signals in the EPR spectrum at $T = 16$ K of *P. furiosus* hydrogenase-I reduced with 10 mM dithionite in the presence of 1 atm CO. Trace (▽) is the normalized (i.e. electronic-gain corrected) amplitude of the negative absorption like feature at $g \approx 1.89$ and has a half-maximal intensity at $P_{0.5} > 0$ dB. Trace (▲) is the amplitude of the central derivative feature at $g \approx 1.93$ with $P_{0.5} = -11$ dB. The nominal microwave output power corresponding to 0 dB attenuation is 200 mW.

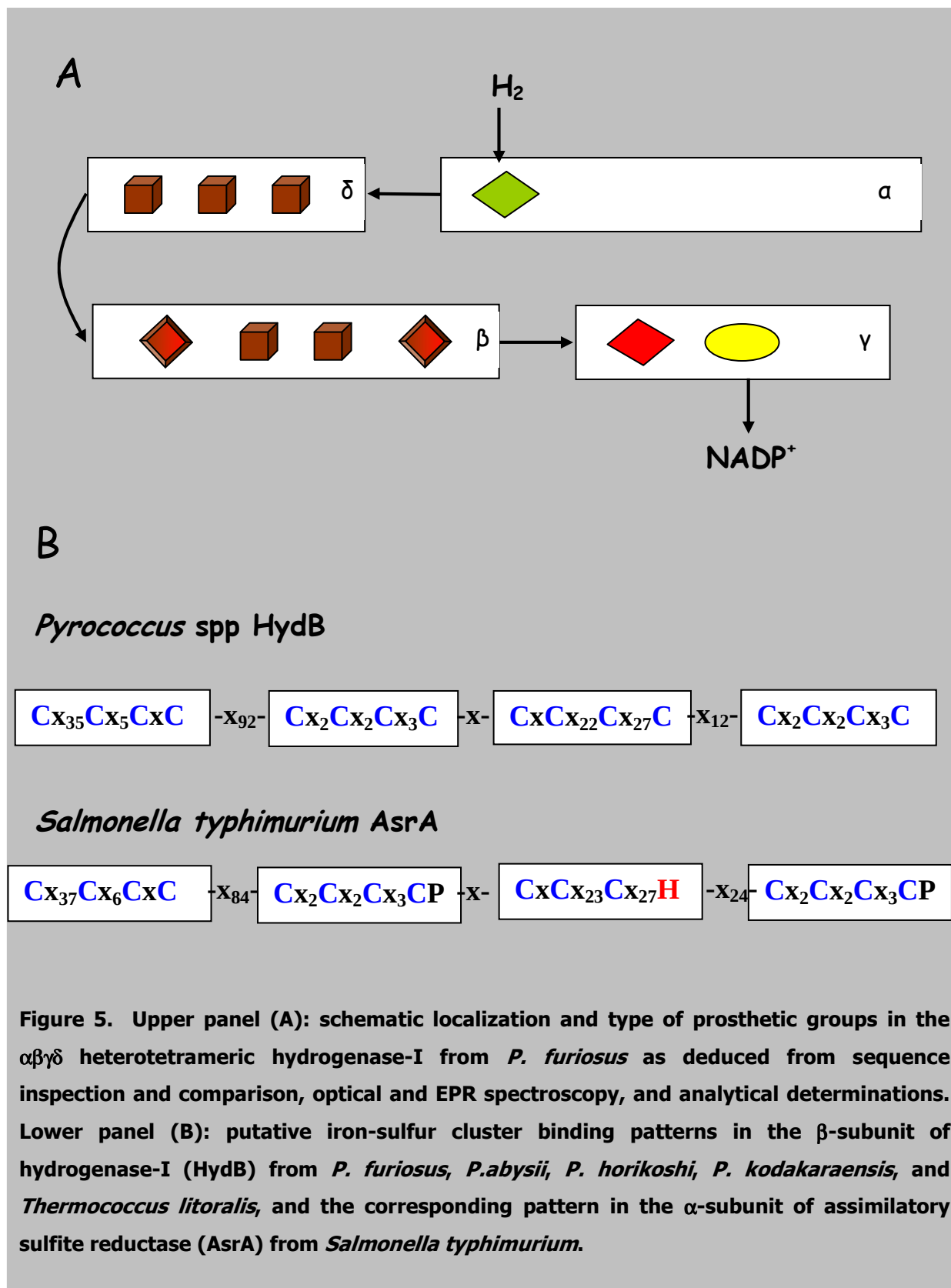
4. Discussion

Figure 5A is an outline of the putative structure and location of four Fe/S clusters in the heterotetrameric soluble hydrogenase-I of *P. furiosus*. The α and δ subunits are homologous to the large and small subunit of many NiFe-hydrogenases, and assignment of the metal cluster binding motifs is straightforward: the H_2 -activating NiFe cluster is coordinated by the α subunit and the δ subunit has cysteine binding motifs for three [4Fe-4S] clusters. Similarly, the γ subunit is homologous to the canonical Spinach ferredoxin plus NADP⁺-dependent reductase (FNR) system and to many similar proteins, and it should therefore, carry a [2Fe-2S] cluster plus a flavin for NAD(P)⁺ cofactor activation. Indeed, hydrogenase-characteristic nickel signals have been observed in EPR [8], and the (partially) reduced enzyme exhibits a close to stoichiometric, near axial Fe/S signal (component 2 in Table 1; cf [11, 12]) whose broadening sets in only at the high temperature of ≈ 70 K [11] as evidence of a slow spin-lattice relaxation rate typical for [2Fe-2S] clusters. Assignment of metal binding motifs to the β subunit is not straightforward: this protein is homologous to the AsrA subunit of the assimilatory sulfite reductase complex from, e.g., *Salmonella typhimurium* [10]. The protein clearly carries Fe/S binding motifs, however, no 3D structure is and also no EPR data are available for this enzyme, therefore, the number and type of Fe/S clusters is uncertain. There are two classical [4Fe-4S] cluster binding motifs CxxCxxCxxxCP, but there is room for one or two more clusters in the form of additional Cys residues, be it in unprecedented motifs (and none comparable to the xanthine oxidase cluster Fe/S II binding motif). With additional information from comparison of putative hydrogenase operons in the genome of *P. furiosus* related species the picture presented in Figure 5 emerges. In the equivalent sequence of sulfite reductase the last Cys in the third binding motif is apparently substituted with His (Figure 5B).

All in all the sequence inspection suggests that there are two regular [4Fe-4S] clusters and presumably two more [4Fe-4S] and/or [2Fe-S] clusters with unusual binding motifs in the β subunit. Together with the three putative [4Fe-4S] clusters in the ‘hydrogenase small subunit like’ δ subunit the enzyme is expected to contain between one and three 2Fe ferredoxin plus between seven and five 4Fe cubane ferredoxin type clusters. A four-component simulation of the EPR spectrum from CO-inhibited, dithionite reduced enzyme

reaches the limits of spectral resolution: in the low-field region only three separate g_z peaks are resolved, and any simulation with more than three components relies on improving the overall fit by ‘filling-in’ minor deviations in between these peaks and in the g_{xy} area while maintaining approximate stoichiometry of each individual component. Combined with the difficulty of accurately doubly integrating these type of wide and tailing spectra, these results afford the final conclusion that the EPR analysis shows the presence of three, or possibly four, reduced ferredoxin centers which is very generously within the limit of eight centers set by sequence analysis.

Since its initial characterization [11] the soluble hydrogenase-I of *P. furiosus* has been ascribed a range of unusual properties that would set it apart from other NiFe hydrogenases: (i) a higher H_2 production than oxidation activity [11]; (ii) an unusually high K_M for H_2 [25]; (iii) energetically uphill activity in the formation of H_2 from soluble, reduced cofactor [26]; (iv) insensitivity to CO inhibition [13, 14]. We have systematically explored and subsequently falsified all these proposals (cf Chapter 2-4 of this thesis), the last one as a subject in the present work. Our final picture of the *P. furiosus* soluble $\alpha\beta\gamma\delta$ hydrogenase-I now summarizes as follows: the enzyme is a hyperthermophilic H_2 : $NADP^+$ oxidoreductase (EC 1.12.1.3) complex. The hydrogen activating part (subunits $\alpha\delta$) is a garden-variety, H_2 consuming NiFe-hydrogenase; the cofactor activating part (subunit γ) is a common FNR-like, NADPH generating enzyme; the electron-transferring β subunit uses a collection of ferredoxin-like clusters to wire the two enzymes together in a single structure. The only remaining enigmatic feature of this system is the elusiveness of approximately half of the iron-sulfur clusters in, e.g., EPR spectroscopy. But then again, EPR silence, presumably as a result of very low reduction potentials, has several precedents in complex iron-sulfur flavin enzyme systems with two distant active sites, e.g, glutamate synthase, dihydropyrimidine dehydrogenase, sulfide dehydrogenase [27, 28, 29, 30].



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Summary

Hydrogenase is an “electron-transfer” protein and catalyses the simplest chemical redox reaction, the reversible two-electron oxidation of molecular hydrogen in aerobic and anaerobic microorganisms, according to the following reaction:



Most known hydrogenases can catalyze the reaction in both direction in vitro, but they usually operate in vivo either in the hydrogen uptake or in the evolution reaction, depending on the demands of the host organism. All hydrogenases contain metal atoms. Most hydrogenases contain Ni atoms, and they all contain several Fe atoms, most of the Fe being organized in Fe-S clusters. The hydrogenases can be divided into two major groups based on their metal contents, the NiFe- and Fe-hydrogenases. Another possible classification is based on their biological function, hydrogen consumption, hydrogen production and bidirectional hydrogenases. Although hydrogenases catalyze a very simple reaction, they do so in many different metabolic contexts and thus assume very different functions. The Fe-hydrogenases are often associated with hydrogen production and the NiFe-hydrogenases with hydrogen consumption. Reported K_M values for hydrogen appear to be consistent with this classification, where Fe-hydrogenases have higher K_M values than NiFe-hydrogenases (Chapter 1).

The structure of the active sites of NiFe-hydrogenases and Fe-hydrogenases are similar in that they encompass a dinuclear metal site with cyanide and carbon monoxide ligands to iron. Any functional directionality is not obvious from the structures. Steady-state kinetic results have been collected using a variety of assay methods and conditions and with enzymes at different stages of purification, which makes it a hazardous undertaking to compare literature values for basic entities, e.g. k_{cat} and K_M .

A re-determination of the K_M (H_2) of the NiFe-hydrogenase-I from *Pyrococcus furiosus* and the K_M (H_2) of the Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) shows, that the values of both hydrogenases are an order-of-magnitude less than literature values. It is proposed that the affinity for hydrogen has been underestimated in previous

studies and that the kinetics of hydrogen activation in relation to the directionality of hydrogenases should be re-evaluated and reanalyzed preferably using purified enzymes under standardized conditions. This will provide a data set of reference for a subsequent endeavor into the fundamental and practical question of the relation between enzyme structure and directional function of hydrogenases (Chapter 2).

The question “where does the substrate bind” is a recurring theme in the biological activation of diatomics such as H_2 , CO and N_2 , whose simple structures are in sharp contrast with that of their biocatalysts. These complex proteins have metal cluster active sites that are unusual to the extent that even high-resolution protein crystallographic data do not readily afford insight into the ways of binding interaction in enzyme-substrate intermediates. The characterization of substrate binding has been hampered by several factors: (i) H is elusive in protein crystallography; (ii) the enzyme-substrate forming reaction is faster than the dead time of the mixing equipment; (iii) purified hydrogenases exhibit a complex activation mechanism with several intermediate states. However, double-resonance data and DFT calculations have been the basis for specific and partially conflicting proposals on side-on or bridging binding modes of hydride or molecular hydrogen to the metal(s) of the NiFe or FeFe active site. In all these proposals it is assumed that maximally only a single hydrogen/hydride interacts with the active site at any time.

A kinetic study of the hydrogen oxidation reaction by Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) reveals a biphasic activation mechanism from as isolated, resting enzyme, via an intermediate state of relatively low activity, to maximally active enzyme. Hydrogen itself is the causative agent for the two subsequent enzyme activation processes. Kinetic-model analysis suggests that the steady-state assumption of Michaelis-Menten kinetics does not apply to this hydrogenase. It appears that activation by hydrogen is cooperative: a catalytic H_2 with high binding affinity is turned over at high rate only after a second, regulatory H_2 with low affinity has been bound to the active site. Hydrogenase activity requires the binding of two separate hydrogen molecules: a positive regulator and a substrate. The proposed novel paradigm of two hydrogen molecules binding simultaneously to the active site may hold for hydrogenases in general (Chapter3).

The soluble NiFe-hydrogenase from *P. furiosus* was originally named “a new type of evolution hydrogenase, because, in contrast to all known NiFe-hydrogenases, hydrogen

production activity at 80°C was found to be higher than the hydrogen consumption activity. Later, it was found that *P. furiosus* contains a membrane-bound hydrogenase with high hydrogen production activity, and this would seem to mean that there is no need for a soluble hydrogen production activity and therefore to leave the soluble hydrogenase without a function. We have therefore re-investigated the steady-state kinetics of the soluble hydrogenase of *P. furiosus*. Our results showed a relatively low K_M for H_2 , which suggests a rather high affinity for hydrogen. We determined that the hydrogen consumption activity was an order of magnitude higher than the hydrogen production activity. These results seem to disqualify the soluble hydrogenase from *P. furiosus* to function as a novel type of hydrogen production enzyme, but they rather suggest that this enzyme is functionally similar to its NAD-reducing soluble multimeric NiFe-hydrogenase congener in *Ralstonia eutropha* with a $K_M = 37 \mu M$ for H_2 . The soluble *P. furiosus* hydrogenase has $K_M \approx 37 \mu M$ for $NADP^+$. We conclude that the soluble hydrogenase from *P. furiosus* is likely to function in the regeneration of NADPH thereby re-using the hydrogen produced by the membrane bound hydrogenase in proton respiration (Chapter 4).

The active center of hydrogenases is always constructed following a single design: a cofactor, based on a sulfur-bridged (Fe,Fe) or (Fe,Ni) pair of metal ions with CO, CN^- ligands on the Fe, close to a [4Fe-4S] cubane for electron transfer. In contrast to this unifying topology electron transfer beyond the first cubane is variable to a considerable degree in structure, function, and compartmentalization, with system complexity ranging from a soluble single subunit hydrogenase in direct electron exchange with a soluble redox partner [Forestier2003], to a hydrogenase that is part of a multi-subunit, multi redox group trans-membrane complex with proton pumping capacity. The heterotetrameric, soluble NiFe-hydrogenase-I of *Pyrococcus furiosus* contains a complex chain of iron-sulfur prosthetic groups for electron transfer between the H_2 -activating NiFe site and the coenzyme-activating flavin. Previous EPR studies have identified only two Fe-S signals in reduced enzyme. In contrast to previous reports the H_2 production activity is found to be sensitive to carbon monoxide with 99% inhibition in CO saturated buffer at 80 °C. Incubation with dithionite under CO at ambient temperature essentially abolished H_2 production and affords stable, maximally reduced enzyme, whose EPR analysis reveals three, possibly four, different Fe-S components. In addition to two subspectra previously assigned to a $[2Fe-2S]^+$ and a high-

potential $[4\text{Fe-4S}]^+$ cluster, there is a third, very anisotropic subspectrum reminiscent of the atypical spectrum of the Fe/S II center in xanthine dehydrogenase. Sequence analysis reveals no homology to this enzyme, but suggests the presence of one $[2\text{Fe-2S}]$, five $[4\text{Fe-4S}]$, and two unprecedented Fe/S binding motifs. The EPR silence of approximately half of the Fe/S clusters under optimized reducing conditions makes this hydrogenase another member of the group of multiheteromeric iron-sulfur flavin enzymes with Fe/S clusters of elusive redox properties (Chapter 5).

Samenvatting

Hydrogenase is een “electron-transfer” eiwit en katalyseert de eenvoudigste redoxreactie, de reversibele twee-electronoxidatie van moleculaire waterstof in aerobe en anaerobe micro-organismen, volgens de volgende reactievergelijking:



De meeste bekende hydrogenases kunnen de reactie in vitro in beide richtingen katalyseren, maar ze functioneren in vivo meestal óf in de waterstofconsumptie óf in de waterstofproductie richting, afhankelijk van het micro-organisme. Alle hydrogenases bevatten metaalatomen. De meeste hydrogenases hebben Ni atomen en allemaal hebben ze enkele Fe atomen, waarbij de meeste Fe atomen zich in ijzer-zwavelclusters bevinden. De hydrogenases kunnen in twee grote groepen worden verdeeld, gebaseerd op aanwezige metaalatomen, de NiFe-hydrogenases en Fe-hydrogenases. Een andere mogelijke verdeling is gebaseerd op de biologische functie van de hydrogenases, waterstofconsumptie-, waterstofproductie- en de bidirectionele hydrogenases. Hoewel hydrogenases een zeer eenvoudige reactie katalyseren, doen ze dat in veel verschillende metabole contexten en vervullen ze dus verschillende functies. De Fe-hydrogenases worden meestal geassocieerd met waterstofproductie en NiFe-hydrogenases met waterstofconsumptie. Gerapporteerde K_M waarden voor H_2 blijken met deze verdeling overeen te komen, waar Fe-hydrogenases hogere K_M -waarden hebben dan NiFe-hydrogenases (Hoofdstuk 1).

De structuur van de actieve centra van NiFe- en Fe-hydrogenases zijn vergelijkbaar omdat ze beiden een dinucleair metaalcentrum hebben, dat is omringd met cyanide- en koolmonoxideliganden. De fysiologische reactierichting is niet afhankelijk van de structuur. Steady-state kinetische resultaten zijn met verschillende analysemethoden verzameld, onder verschillende condities en met enzymen van verschillende zuiverheden, waardoor het moeilijk is om literatuurwaarden als k_{cat} en K_M met elkaar te vergelijken.

Een herbepaling van de K_M (H_2) van hydrogenase-I van *Pyrococcus furiosus* en de K_M van de Fe-hydrogenase van *Desulfovibrio vulgaris* (Hildenborough) laat zien, dat de waarden van beide hydrogenases een factor 10 lager zijn dan de literatuurwaarden. Het is

waarschijnlijk dat de affiniteit voor waterstof in eerdere studies is onderschat en dat de kinetiek van waterstofactivering in relatie tot de reactierichting opnieuw moet worden bepaald en geanalyseerd, bij voorkeur met zuivere enzymen en onder standaard condities. Dit zal een set referentiedata geven om in een volgende stap de fundamentele en praktische vraag te beantwoorden, wat de relatie is tussen de enzymstructuur en de directe functie van hydrogenases (Hoofdstuk 2).

De vraag “waar bindt het substraat” is een terugkerend thema in de biologische activering van diatomaire moleculen als H_2 , CO en N_2 , waar de simpele structuur in scherp contrast staat met die van hun biokatalysatoren. Deze complexe eiwitten hebben in de actieve centra zulke ongewone metaalclusters, dat zelfs eiwitkristallografiedata met hoge resolutie geen inzicht geven in de bindingsinteractie in enzym-substraat intermediären. De karakterisering van substraatbinding wordt bemoeilijkt door verschillende factoren: (i) H is moeilijk te vinden in eiwitkristallografie; (ii) de enzym-substraat vormende reactie is sneller dan de “dead-time” van de mengapparatuur; (iii) gezuiverde enzymen vertonen een complex activeringsmechanisme met verschillende intermediaire toestanden. Desalniettemin vormen, dubbel-resonantie data en DFT berekeningen de basis voor specifieke en deels conflicterende hypothesen voor “side-on” of “bridging” bindingen tussen hydride of moleculair waterstof en het metaal of de metalen van het NiFe- of FeFe-actieve centrum. Er wordt in al deze hypothesen vanuit gegaan dat op elk moment maximaal één enkele waterstof of hydride interactie vertoont met het actieve centrum.

Een kinetische studie van de waterstofoxidatiereactie door het Fe-hydrogenase van *D. vulgaris* (Hildenborough) geeft een tweefasig activeringsmechanisme van een geïsoleerd “rustend” enzym, via een intermediaire toestand met relatief lage activiteit, tot een maximaal actief enzym. Waterstof zelf is de activator in de twee opeenvolgende enzymatische activeringsprocessen. Kinetische modelanalyse suggereert, dat de aanname van een steady-state toestand uit de Michaelis-Menten kinetiek niet geldt voor dit hydrogenase. Het blijkt, dat activering door waterstof coöperatief is: een katalytische H_2 met een hoge bindingsaffiniteit reageert met een hoge snelheid, alleen wanneer een tweede, regulerende H_2 met een lage bindingsaffiniteit aan het actieve centrum is gebonden. Hydrogenase-activiteit vereist de binding van twee verschillende waterstofmoleculen: een positieve regulator en een substraat.

Het nieuwe paradigma van twee waterstofmoleculen die tegelijk binden aan het actieve centrum geldt mogelijk voor hydrogenases in het algemeen (Hoofdstuk 3).

Het oplosbare NiFe-hydrogenase van *P. furiosus* werd oorspronkelijk een “nieuw type productie-enzym” genoemd, omdat de waterstofproductie-activiteit bij 80°C, in tegenstelling tot bij alle bekende hydrogenases, hoger was dan de waterstofconsumptie-activiteit. Later ontdekte men, dat *P. furiosus* een membraangebonden hydrogenase met hoge waterstofproductie-activiteit bevat. Dit zou betekenen, dat er geen behoefte is aan een oplosbare waterstofproductie-activiteit waardoor het oplosbare hydrogenase geen functie zou hebben. Daarom hebben we de steady-state kinetiek van het oplosbare hydrogenase van *P. furiosus* opnieuw bestudeerd. Onze resultaten geven een zeer lage K_M voor H_2 , wat suggereert dat er een hoge affiniteit voor waterstof is. We ontdekten, dat de waterstofconsumptie-activiteit ongeveer een factor 10 hoger is dan de waterstofproductie-activiteit. Het lijkt er dus op, dat het oplosbare hydrogenase van *P. furiosus*, niet een nieuw soort waterstofproducerend enzym is. De resultaten suggereren, dat dit enzym functioneel vergelijkbaar is met NAD-reducerende, oplosbare, multimere NiFe-hydrogenase in *Ralstonia eutropha* met een K_M van 37 μM voor H_2 . Het oplosbare hydrogenase van *P. furiosus* heeft een K_M van ongeveer 37 μM voor $NADP^+$. We concluderen, dat het oplosbare hydrogenase van *P. furiosus* blijkbaar een functie heeft in de regeneratie van NADPH, waarbij waterstof, geproduceerd door het membraangebonden hydrogenase tijdens protonrespiratie, wordt her-gebruikt (Hoofdstuk 4).

Het actieve centrum van hydrogenases is altijd opgebouwd volgens een algemeen stramien: een cofactor gebaseerd op een zwavel-gebrugd (Fe, Fe) of (Ni, Fe) paar van metaalionen, met CO en CN^- liganden die aan het Fe zijn gebonden, dicht bij een [4Fe-4S] cluster voor electronentransport. In tegenstelling tot deze standaard-topologie is electronentransport na deze eerste cubaan in sterke mate variabel in termen van structuur, functie en compartimentalisatie, waarbij de complexiteit loopt van van een oplosbare hydrogenase bestaande uit een enkele subunit met een directe electronenuitwisseling met een oplosbare redoxpartner, tot een hydrogenase, dat onderdeel is van een multisubunit, multiredox transmembraancomplex dat functioneert als protonpomp.

Het oplosbare heterotetramere NiFe-hydrogenase-I van *Pyrococcus furiosus* bevat een complexe keten van ijzer-zwavel prosthetische groepen voor electronentransport tussen het waterstof-activerende NiFe centrum en de co-enzym-activerende flavine. In eerdere EPR

studies zijn slechts twee Fe-S signalen in het gereduceerde enzym geïdentificeerd. In tegenstelling tot eerdere rapporten, is de H₂ productie-activiteit gevoelig voor koolmonoxide, met een remming van 99% in CO-verzadigde buffer bij 80°C. Incubatie met dithioniet onder CO bij kamertemperatuur stopt de H₂ productie en geeft een stabiel en maximaal gereduceerd enzym, dat in EPR-analyse drie, of mogelijk vier verschillende Fe-S componenten laat zien. Naast twee subspectra, eerder toegeschreven aan een [2Fe-2S]⁺ en een hoge potentiaal [4Fe-4S]⁺ cluster, is er een derde, zeer anisotroop subspectrum dat lijkt op een ongewoon spectrum van het Fe/S II centrum in xanthine hydrogenase. Sequentie-analyse laat geen homologie zien met dit enzym, maar suggereert de aanwezigheid van één [2Fe-2S], vijf [4Fe-4S], en twee Fe/S clusters van een niet eerder gevonden type. De afwezigheid van EPR signalen voor ongeveer de helft van de Fe/S clusters onder optimaal reducerende omstandigheden maakt dit hydrogenase een nieuw lid van de groep van multiheteromere ijzer-zwavel flavine enzymen met Fe/S clusters met afwijkende redoxeigenschappen (Hoofdstuk 5).

Curriculum Vitae

Daan van Haaster was born in Rotterdam on May 15, 1975. He completed his secondary education (VWO) at the Emmauscollege of Rotterdam in 1994. In the same year he initiated his studies in Chemistry at the University of Utrecht. His final research project was in the Bio-Organic Chemistry group of prof. dr. J.F.G. Vliegthart, and he graduated in August 2000. In September 2000 he started in the “Postgraduate study Biotechnology” joint curriculum at Delft University of Technology and Leiden University. His second-year research project was in the Delft Enzymology group of prof. dr. W.R. Hagen, and he graduated in November 2003 as ‘Master of Biotechnology’ with specialization ‘Process Biochemistry’. The research project coincided with the first year of a PhD research under supervision of prof. dr. W.R.Hagen, which resulted in the present thesis. From May, 2006 he is employed as a Post-Doc in the Food Chemistry group of prof. dr. ir. A.G.J. Voragen at Wageningen University where he is working in the Healthgrain project on novel technologies to produce nutritionally optimized food from cereal grains and to get insight in process-induced changes in bioactive compounds.

List of Publications

1. **van Haaster, D.J.**, Hagedoorn, P.-L., Jongejan, J.A. and Hagen, W.R. (2007) The Intramolecular Electron Transfer Chain of the Soluble, NADPH-dependent Hydrogenase-I of *Pyrococcus furiosus*, *in preparation*.
2. **van Haaster, D.J.**, Silva, P.J., Hagedoorn, P.-L., Jongejan, J.A. and Hagen, W.R. (2007) A Re-Investigation of the Steady-State Kinetics of the Soluble NiFe-Hydrogenase-I from *Pyrococcus furiosus*, *submitted*.
3. Voronin, O.G., **van Haaster, D.J.**, Karyakina, E.E., Hagen, W.R., Karyakin, A.A. (2007) Direct bioelectrocatalysis by NADP-reducing hydrogenase from *Pyrococcus furiosus*, *submitted*.
4. **van Haaster, D.J.**, Jongejan, J.A., Hagedoorn, P.-L. and Hagen, W.R. (2006) Biological Hydrogen Activation is Cooperative: One H₂ Activates the Dinuclear Center and a Second H₂ is Oxidized. *Int. J. Hydrogen Energy* **31**, 1432-1438.
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