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Structural bases for the catalytic mechanism of NiFe hydrogenase

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Introduction

The reaction $H_2 <----> 2H^+ + 2e^-$ provides electrons at a significantly low potential (the H2/2H+ redox couple has a E° of -414 mV at pH 7 and 1 bar of H_2). However, hydrogen oxidation is not energetically favourable because of the very low acidity of the H-H bond (pKa = 35). This can be dramatically changed through binding of molecular hydrogen to a metal center since the pKa can be lowered by as many as 20 to 30 units, (ref.1, 2). This, in turn, facilitates the heterolytic cleavage of the H_2 molecule (giving $H^- + H^+$), a first step in the oxidation process. Hydrogen metabolism is present in a wide variety of chemotrophic and phototrophic microorganisms where hydrogen consumption or production are central features of such various processes as biogas production, denitrification, nitrogen fixation, corrosive sulfate reduction, production of H_2 from water and specific hydrogenation reactions or cofactor regeneration (ref. 3,4).

The enzymes responsible for H_2 metabolism are called hydrogenases and have been classified as [NiFe] and [Fe]-only enzymes (ref. 4). [NiFe] hydrogenases are heterodimeric with relatively well conserved 60kDa large subunits and more diverse small subunits (ref. 5) containing one or more 4Fe4S clusters (Fig. 1). It has been shown that hydrogen activation is mediated by the nickel center and that the iron-sulfur clusters are probably involved in electron transfer between the active site, the molecular surface and the redox partners of the enzyme, such as cytochromes and ferredoxins (ref. 5).

EPR studies

[NiFe] hydrogenases have been subjected to extensive EPR studies (see ref. 5 and references therein). Three paramagnetic Ni states, Ni-A, Ni-B and Ni-C, have been identified which depend on O_2 and the redox potential. Ni-A and Ni-B represent the EPR spectra produced by the most oxidized states. Neither of these forms seems to be part of the catalytic cycle which operates at significantly more negative potentials (ref. 6). The Ni-C signal corresponds to a partially reduced form with a bound hydrogen species supposed to be either H_2, H^+ or H^- (ref. 5). The binding of a hydrogen species to the active site Ni center has been demonstrated by the isotopic effect induced by deuterium on the photolysis of Ni-C, as detected by EPR (ref. 7). Ni-C seems to be the only stable paramagnetic species which is part of the catalytic cycle.
Redox titrations of *D. gigas* hydrogenase (ref. 6) have indicated that besides Ni-A/B and Ni-C, the active site can exist in two other EPR-silent states, NI-SI and Ni-R. The various redox states are separated by sequential one-electron reductions in the following order: Ni-B --> Ni-SI --> Ni-C --> Ni-

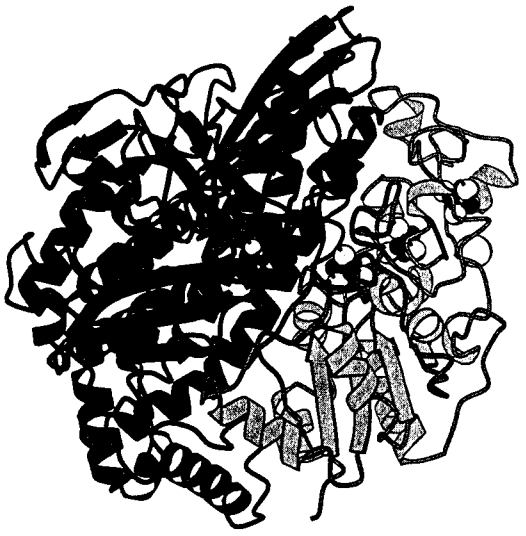


Figure 1. Overall picture of the fold of the [NiFe] hydrogenase of *Desulfovibrio gigas*. Ribbons and arrows designate α-helices and β-strands, respectively. The large subunit is shown in dark grey, the small subunit in light gray. The latter binds one [3Fe-4S] and two [4Fe-4S] clusters. The color code for the cofactors is: black Fe, white Ni inorganic S.

Redox titrations

R. These and other studies (ref. 7) have led to the proposition of a cyclic catalytic mechanism involving Ni-SI (as the active H2 oxidant), Ni-C, and Ni-R (as the active H+ reductant). In addition to the active site redox center, *D. gigas* hydrogenase has two [4Fe-4S] clusters with midpoint redox potentials close to those of the Ni-SI/Ni-C and Ni-C/Ni-R couples, respectively. A full description of any overall state of this hydrogenase has to be expressed as a combination of the states of the active site and the two [4Fe-4S] clusters. This results in a complex combination of twelve possible microstates, forming three cycles, for which relative populations have been determined at various potentials (ref. 6).

A simpler scheme, containing six microstates, can be proposed if only one [4Fe-4S] cluster is considered (which may be the case in some [NiFe] hydrogenases (ref. 5, 8). This minimal scheme contains two Ni-SI --> Ni-R --> Ni-C --> Ni-SI cycles (Fig. 2). Cycle I, which can only oxidize H2, seems to operate at potentials that are too positive for hydrogenase redox partner, the cytochrome c3 (E = -290 mV at pH 7). This suggests that either such cycle is not biologically relevant (except the S2 ---> R2 passage that provides access from cycle I to cycle II), or that, at low P(H2), there may exist hydrogenase electron acceptors with a more positive redox potential than cytochrome c3. On the other hand, Cycle II, operating at more reduced potentials, can both oxidize H2 and reduce protons. Upon H2 removal by flushing with an inert gas, two isoelectronic species (belonging to cycles I and II) co-exist (ref. 6): a Ni-C species with an oxidized FeS cluster and a Ni-SI state with a reduced one (C2 and S1, respectively, see Fig. 2).

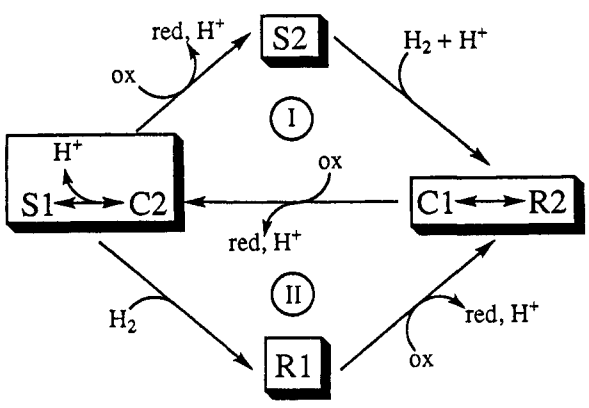


Figure 2. Simplified scheme, based on the work of Roberts and Lindahl (ref. 6) of possible H_2 oxidation cycles in a "minimal" [NiFe] hydrogenase containing only one [4Fe-4S] cluster (the one proximal to the active site, see Fig. 1). S, C and R refer to subsequently more reduced states of the active site, whereas 1 and 2 label the oxidation state of the [4Fe-4S] cluster. Iso-electronic states are boxed. The external redox partner is denoted by ox and red. Only cycle II can operate in both directions.

These results have been discussed assuming that the active site of the [NiFe] hydrogenases was a mononuclear Ni center capable of performing both catalysis (H-H bond splitting) and redox chemistry (electron transfer) (ref. 5-7,9). There is little doubt that the Ni center constitutes the hydrogenase catalytic site, however, the assumption of Ni-based redox chemistry is more problematic. On the one hand, X-ray absorption (XAS) experiments have shown that the electron density at the Ni center does not change significantly as a function of the various redox states (ref. 10). On the other, the redox changes that the Ni center is supposed to undergo, namely Ni(III) *(Ni-A/B)* -->Ni(II) *(Ni-SI)* -->Ni(I) *(Ni-C)* --->Ni(0) *(Ni-R)*, (ref. 5) are difficult to reconcile with the results from model chemistry which indicate that, for instance, the Ni(III)/Ni(II) and Ni(II)/Ni(I) redox couples are too far apart to be accomodated within the range of observed redox potentials in hydrogenases (ref. 11).

Crystallographic results and IR spectroscopy.

Our X-ray crystallographic analysis of *D. gigas* [NiFe] hydrogenase has revealed that the active site is, in fact, binuclear with a second metal center, that we have shown to be iron, located at less than 3 Å from the nickel ion (figs. 1 and 3 and ref. 12, 13). The Ni ion binds only four protein ligands. Cysteines 65 and 530 are terminal ligands whereas cysteines 68 and 533 bridge the two metals centers. We have also shown that the Fe center binds three non-protein diatomic ligands (Fig. 3 and ref. 13). The discovery of such an unexpected Ni-Fe center has deep implications for the understanding of biological H2 catalysis and requires a re-interpretation of the relevant available data. For instance, the existence of a binuclear center provides an alternative to a Ni ion which would be both catalytic and extensively redox active. Since the second metal site is occupied by a Fe ion, then at least some of the redox chemistry could take place at this site. Indeed, recent studies on the FTIR analyses of *Chromatium vinosum* and *D. gigas* hydrogenases (ref. 13, 14) have shown the presence of three intrinsic high frequency IR bands normally absent from protein spectra. Furthermore, it was observed that the three bands shifted, in a concerted fashion, as a function of the redox state of the enzyme. It is almost certain that the IR bands ion, which have now been assigned to one CO and two CN-'s (ref. 15), arise from the three non-protein ligands of the active site Fe. Consequently, they report on redox changes being sensed by this center.

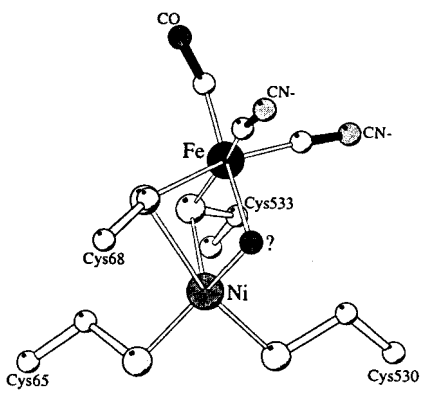
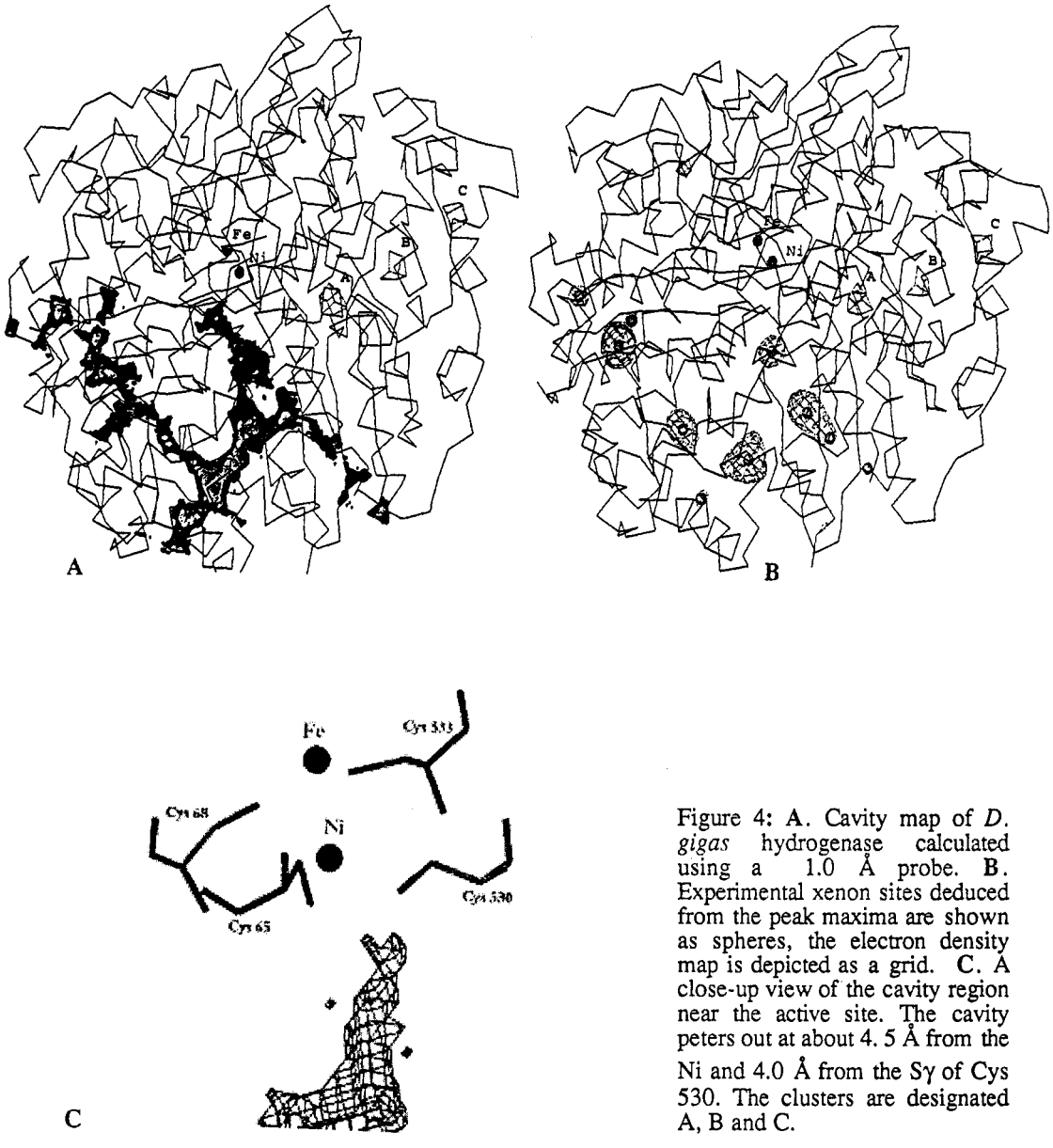


Figure 3. The heterobinuclear active site of *D. gigas* hydrogenase. The Fe center has three ligands: one CO and two CN-'s. The Ni is ligated by four thiolates, two of which also bind to the Fe. The question mark designates a ligand (probably oxygen) that bridges the two metal centers in the oxidized enzyme. The Ni coordination es distorted square pyramidal; the empty axial site is trans to the Sγ of Cys 533.

Gas accessibility to the active site.

In order to locate possible H2 transfer pathways between the active site and the molecular surface, a cavity map was calculated from the model of *D. gigas* hydrogenase using a probe radius of 1.0 Å (Fig. 4A). Since all observed ordered solvent molecules were included in the model, only internal hydrophobic cavities at least 2.0 Å in diameter were selected. The result is a network of hydrophobic channels whose shape roughly resembles the letters V and A connected through their right and left sides, respectively (Fig. 4A). A channel starting at the interface between the small and the large subunit of hydrogenase extends from the bottom of the V motif to the protein surface. The left arm of the V is surrounded entirely by residues of the large subunit and shows several invaginations pointing towards the bulk solvent. The right leg of the A, which is located in the N-terminal domain of the small subunit, provides another possible access for gases to the active site. From this analysis, it appears that



A B C

Figure 4: A. Cavity map of *D. gigas* hydrogenase calculated using a 1.0 Å probe. B. Experimental xenon sites deduced from the peak maxima are shown as spheres, the electron density map is depicted as a grid. C. A close-up view of the cavity region near the active site. The cavity peters out at about 4. 5 Å from the Ni and 4.0 Å from the Sγ of Cys 530. The clusters are designated A, B and C.

there are several putative entrance sites for gas diffusion connected by different channels to a unique entrance to the active site.

To probe gas accessibility to these hydrophobic channels, xenon binding experiments were carried out using crystals of the Ni-Fe hydrogenase of *Desulfovibrio fructosovorans* , which shows 65% amino acid sequence identity with the *D. gigas* enzyme (ref. 16). Given the hydrophobic nature and high atomic number of Xe, its binding is a convenient way to investigate the gas accessibility of protein interiors by crystallographic methods (refs. 17,18). X-ray diffraction data for the Xe derivative of *Desulfovibrio fructosovorans* hydrogenase was prepared using a high-pressure capillary set-up. Ten major peaks were found (Fig. 4B), showing heights ranging from 4.1 to 8.3 times the root mean square (rms) value of the map. The protein environment of each of the Xe sites is highly hydrophobic and 85% of the amino acid residues within 4.5 Å from these sites are identical in *D. gigas* and *D. fructosovorans* hydrogenases.

The end of the hydrophobic channel that approaches the active site is about 4.5 Å from the Ni ion and points at its empty axial coordination site. Cys 530 (that is substituted by a selenocysteine in some hydrogenases; ref. 19) is also close to this channel and could play the role of a base in the heterolytic cleavage of molecular hydrogen (Fig. 4C). Thus, our cavity analysis provides indirect support for Ni being the primary H2 binding site through its vacant axial coordination site.

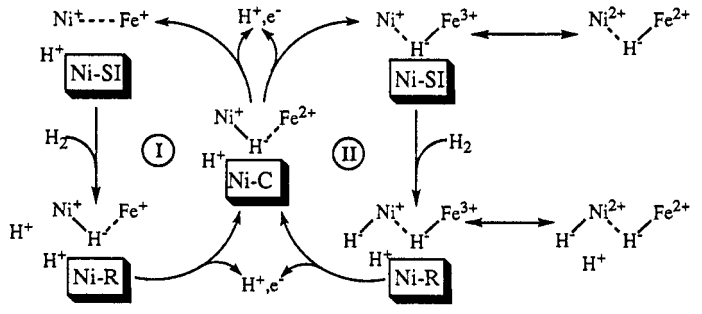


Figure 5. Possible H_2 oxidation cycles (marked I and II) in *D. gigas* hydrogenase. The central assumption is that the bridging putative oxygen species observed in the crystal occupies the position of a hydride in the Ni-C state (see text). States shown at the same level are both isoelectronic and isoprotonic. For simplicity, only the active site metal ions and those hydrogen species that may be directly involved in catalysis are shown.

Model Chemistry

The active site of [NiFe] hydrogenases has been extensively modelled (for a recent review see ref. 20) Of primary importance are those compounds that can shed light on the influence of the coordination sphere and valence state of the Ni ion on the catalytic properties of hydrogenases. Studies by the groups of Holm (11), Darensbourg (21) and Mascharak (1) have highlighted the importance of S-ligands in the coordination sphere of the Ni ion. Both sulfur coordination and a Ni(I) redox state seem to be requirements for H2 activation and subsequent hydride formation. Although these studies have focused on mononuclear Ni compounds, their results concerning hydrogen binding and activation should apply to the binuclear active site of hydrogenases since the Ni ion is very likely to constitute the catalytic site in these enzymes.

Catalytic mechanism

From the recent data on the structure and function of [NiFe] hydrogenase it is possible to propose two plausible schemes for hydrogen biocatalysis. Based on the minimal scheme proposed above (fig. 2) the process can be divided into 4 different steps.

1) H_2 binding : Several lines of evidence suggest that the primary binding site of molecular hydrogen is the Ni center. At relatively high potentials, the most likely candidate would be a Ni-SI state (ref. 6). However, if this is a Ni(I) species (see above), then the two metals at the active site must be coupled in order to generate a diamagnetic center. A likely state for Ni-SI would then be Ni(I)-Fe(I). At more reduced potentials, the H_2 binding species could be Ni-C with an oxidized FeS cluster (ref. 9). Since the Ni-C state is one electron more reduced than the Ni-SI state and paramagnetic, a plausible electronic state for this species is Ni(I)-Fe(II) with a bound hydride. We have suggested that H^- could bridge the two metal centers (ref. 12). Substrate binding and heterolytic cleavage by a Ni-C species would form a double hydride (fig. 5). This is possible since the Ni coordination in the putative Ni-C form wolud be distorted square pyramidal and, consequently, there would be an available ligand site. H_2 (followed by H^-) binding to the vacant site would make the Ni coordination transiently octahedral (see **3)** below).

2) H_2 cleavage : From the hydrogenase active site geometry and our Xe binding and H_2 diffusion simulations (see above) it can be assumed that, in any case, H_2 binds to the vacant axial coordination site (Fig. 3). If this is the case, the Cys530 thiolate is a good candidate for the base that would bind the proton resulting after heterolytic cleavage, whereas the newly formed hydride would have different fates depending on which is the binding Ni species.

3) H^- binding : If the primary oxidant is Ni-SI (ref. 6) then the hydride would move to a bridging position and the resulting Ni-R species (Fig. 5) could be Ni(I)-Fe(I) with a bridging hydride. If, on other hand, the oxidant is a Ni-C state with one oxidized FeS cluster, a transient species would form, three electrons more reduced than Ni-SI. This species would have a bridging hydride and a terminal one bound to the primary substrate site and a Fe(II)-Ni(I) metal redox state. This, or an isoelectronic species, would then give an electron to the FeS cluster to form the Ni-R state.

4) Redox cycling : In both cases, the transfer of one electron from the Ni-R active site to a FeS cluster would (re)generate the Ni-C state. The cycle will be closed either by the subsequent one-electron oxidation of Ni-C to give Ni-SI or by oxidation of the FeS cluster of the Ni-C species (electron transfer towards the redox partner). In scheme 1, the Ni ion may stay as Ni(I) throughout catalysis whereas the Fe ion shuttles between Fe(I) (Ni-SI and Ni-R) and Fe(II) (Ni-C). In scheme 2, the Ni-R could be a double hydride Ni(II)-Fe(II) species. The two schemes are not mutually exclusive and one or the other may operate preferentially depending on the physiological conditions.

Conclusions

Recent structural and functional results have furthered our understanding of H_2 biocatalysis at the molecular level. The presence of a heterobinuclear center in hydrogenases suggests that the catalytic and redox sites may be different. The Ni ion appears as the most likely catalytic site, given the general

characteristics of this metal. In turn, redox activity, as indicated by IR spectroscopy, may be mostly carried out by the Fe center.

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