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## The mechanism of the Ni-Fe hydrogenases: a quantum chemical perspective

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**Abstract** The catalytic cycle for the heterolytic splitting of H<sub>2</sub> by Ni-Fe hydrogenase has been investigated in four recent quantum chemical studies. The mechanisms proposed are described and compared. Although there are clear differences in these mechanisms and in the assignments of the different states observed experimentally, there are also important points of consensus.

**Keywords** Ni-Fe hydrogenase · Mechanism · Theoretical studies · Ni-Fe cluster

In recent years, the continuing advance in the power of quantum chemical techniques has begun to permit reliable application to the problem of enzyme mechanisms. These studies have given insight into the mechanisms of such challenging systems as methane monooxygenase [1, 2] and the oxygen evolving center of Photosystem II [3, 4]. Increased attention was drawn to the hydrogenase problem by the surprising discovery by X-ray crystallography of a bimetallic Ni-Fe cluster at the active site of the Ni-Fe hydrogenases [5, 6], together with the identification by vibrational spectroscopy by Albracht and co-workers [7] of the organometallic Fe(CO)(CN)<sub>2</sub> group as a key component of the cluster.

The hydrogenase enzymes [8] catalyse the interconversion of dihydrogen and protons, H<sub>2</sub> ⇌ 2H<sup>+</sup> + 2e<sup>-</sup>, and allow the use of H<sub>2</sub> as an energy source by bacteria or allow the disposal of excess electrons in the form of

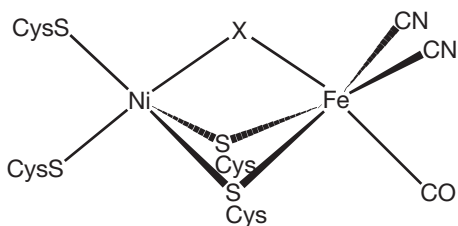
H<sub>2</sub>. Hydrogenases from the sulfate reducing bacterium *Desulfovibrio gigas*, the photosynthetic bacterium *Chromatium vinosum*, and the H<sub>2</sub>-oxidizing bacterium *Alcaligenes eutrophus*, among others, have been studied in detail. Hydrogenases also recapture the reducing power lost in nitrogen fixation by the apparently unavoidable formation of H<sub>2</sub> as by-product in N<sub>2</sub> reduction by nitrogenase. Four main classes of hydrogenases are recognized: the Ni-Fe hydrogenases contain both metals and are the most numerous, followed by the Ni-Fe-Se enzymes which also contain one selenocysteine residue as a ligand for Ni; Fe-only enzymes are also known, and a novel metal-free hydrogenase has recently been found by Thauer et al. [9]. The essential nickel in the Ni-Fe enzymes [10] was significant because Ni is rare in biology [11]. Depending on the source, Ni-Fe hydrogenases contain different numbers of metal clusters, but the Ni-Fe cluster is believed to be the site of H<sub>2</sub> binding; other Fe-S clusters are thought to be involved in electron transfer.

Electron paramagnetic resonance (EPR) data for *D. gigas* H<sub>2</sub>-ase [12, 13] shows several different enzyme states. In air, two catalytically inactive forms are found with different types of EPR signal: Ni-A or Ni<sub>u</sub> (unready form) and Ni-B or Ni<sub>r</sub> (ready form); an EPR-silent form is also known. With H<sub>2</sub>, the Ni-B form rapidly gives the active form that shows the Ni-C EPR signal; reduction of this form yields two further EPR-silent forms, one of them being Ni-R. Ni-A is only activated by H<sub>2</sub> over several hours. Ni-C is photosensitive and below 80 K a change in the EPR to give Ni-L is seen. The strong kinetic isotope effect ( $k_H/k_D=6$ ) suggests H<sub>2</sub> may interact by direct binding with the Ni cluster. The Ni-A, -B, and -C signals all show significant hyperfine coupling to <sup>61</sup>Ni, so they must be associated with the Ni-Fe cluster.

The X-ray crystal structure of the Ni-Fe hydrogenase from *D. gigas* in the inactive air-oxidized form [5] indicates that the larger 60 kDa subunit contains a

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**Fig. 1** A schematic view of the structure of the Ni-Fe cluster of the *D. gigas* hydrogenase from X-ray and IR spectroscopic investigations

Ni-Fe cluster and the smaller 28 kDa subunit contains the Fe-S clusters. The Ni-Fe cluster consists of a Ni bound to four Cys thiolates, two of which are bridging to the Fe. An additional bridge between Fe and Ni is tentatively identified [5] as  $\mu$ -oxo and considered to be associated with the oxidized inactive form of the enzyme, reduction to give the active form being accompanied by loss of this group. The iron center has three terminal ligands which have been identified as two CN and one CO (Fig. 1) [7].

The Ni ion itself may be redox active and able to attain oxidation states Ni(III), Ni(II), and Ni(I), but this has been challenged because the Ni X-ray absorption edge shows no shift between the Ni-A, Ni-B, and Ni-C states, neither for *D. gigas* [14] nor for *Thiocapsa roseopersicina* enzymes [15]. No model compounds have Ni(I)/(II) and Ni(II)/(III) redox potentials so closely spaced as found in the enzyme [16, 17].

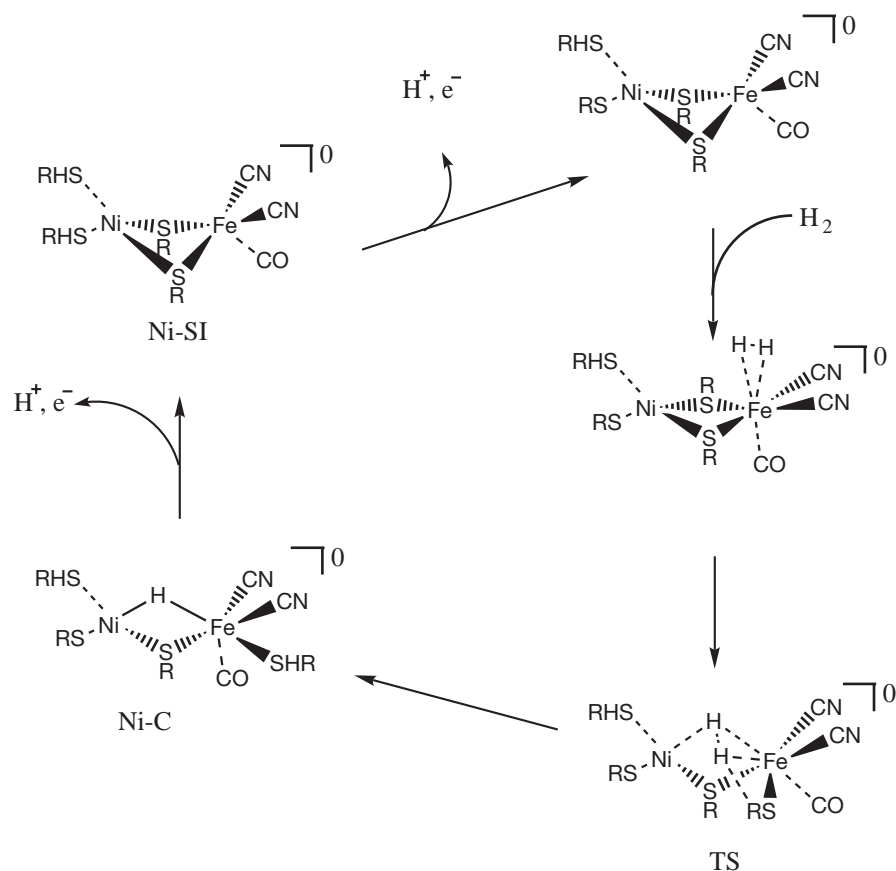
Many mechanistic schemes have been proposed [12], but there is little general agreement. In the first theoretical paper on the mechanism of Ni-Fe hydrogenase [18], quantum chemical methods were used to clarify the situation. Calculations were performed in two steps, using the DFT-B3LYP method for geometry optimization with double zeta basis sets, followed by an energy evaluation with a large basis set. In initial studies, SH groups were used to model the Cys ligands of the enzyme but the real CO and CN ligands were used in full. Neutrally charged clusters were used both for computational simplicity and because zero charge seems reasonable in the low dielectric medium of the protein. The bridging oxygen derived ligand shown in the enzyme structure was removed to model the conversion to the active catalytic state. As in other enzyme systems [1, 2, 3, 4] we seek a series of intermediates and transition states with energies in a slowly descending channel so that a reasonable driving force is maintained in each step around the cycle and no barrier between one intermediate and the next is too high for the system to cycle at the observed catalytic rate. Also, as in other enzyme systems [1, 2, 3, 4], the question of spin states needs attention. In general, a number of states are available to paramagnetic active site clusters and the lowest-barrier route may involve a spin state other than the ground state as well as spin

crossings between different steps. These crossings are probably very fast in real systems of low symmetry and so do not limit the overall rate. The alternative case of slow spin crossing has also been considered, however ([19] and references cited therein)

From the DFT calculations a cycle was proposed where the first intermediate involves hydrogen complexation as a molecular hydrogen complex to the Fe center. This type of binding is known to strongly acidify  $H_2$ , enabling it to act as a proton donor to the basic adjacent SR group. A transition state for the resulting heterolytic cleavage was located. The resulting Fe-bound hydride was subsequently able to migrate to the Ni atom. The subsequent net abstraction of two H atoms, equivalent to  $2H^+ + 2e^-$ , was not considered explicitly in this work.

In another study [20] and in more recent work (Wirstam M, to be published) this proposal has been extended and modified, suggesting the cycle shown in Fig. 2. The main features of this catalytic cycle can be described as follows. Starting in the upper right corner of this figure, a hydrogen molecule becomes bound to the iron center. No similar minimum was found for binding to the nickel center. The next step is the heterolytic cleavage of the hydrogen molecule. Only one possible pathway for the  $H_2$  splitting has been found, which is probably the most significant result of the entire investigation. The splitting occurs on the iron center and involves an NiFe(II,III) complex (EPR active), leading to a metal-bound hydride and a protonation of a bridging cysteine that eventually ends up as a terminal iron ligand. [The symbol NiFe(II,III) is used to indicate that the individual oxidation states on iron and nickel could not meaningfully be assigned solely based on the information from the DFT calculations. The oxidation states could be either Ni(II)Fe(III) or Ni(III)Fe(II).] No corresponding low-energy pathway was found on the nickel center despite numerous attempts. Likewise, no low-energy pathway was found for model complexes with an even number of electrons (EPR-silent complexes). After the transition state, the hydride forms a bridge between Ni and Fe. In the next step the hydride moves over to a terminal cysteine and the proton initially bound to the bridging cysteine is lost to the proton channel of the enzyme. Simultaneously, an electron is transferred from the cluster. The complex formed, which has an even number of electrons and should be EPR silent, is identified as Ni-SI. The formal oxidation state is NiFe(II,II). It should be noted that the present structure of Ni-SI (see Fig. 2) has a quite long Ni-Fe distance and may therefore be inconsistent with the results of EXAFS studies. Further investigations to find alternative structures are necessary to resolve this problem. In the final step the remaining proton and electron from  $H_2$  are removed and the complex returns to its original form. To assign Ni-C we note that it should be EPR active. The most stable structure with an odd number of electrons in

**Fig. 2** Catalytic cycle for Ni-Fe hydrogenase slightly modified from [20]

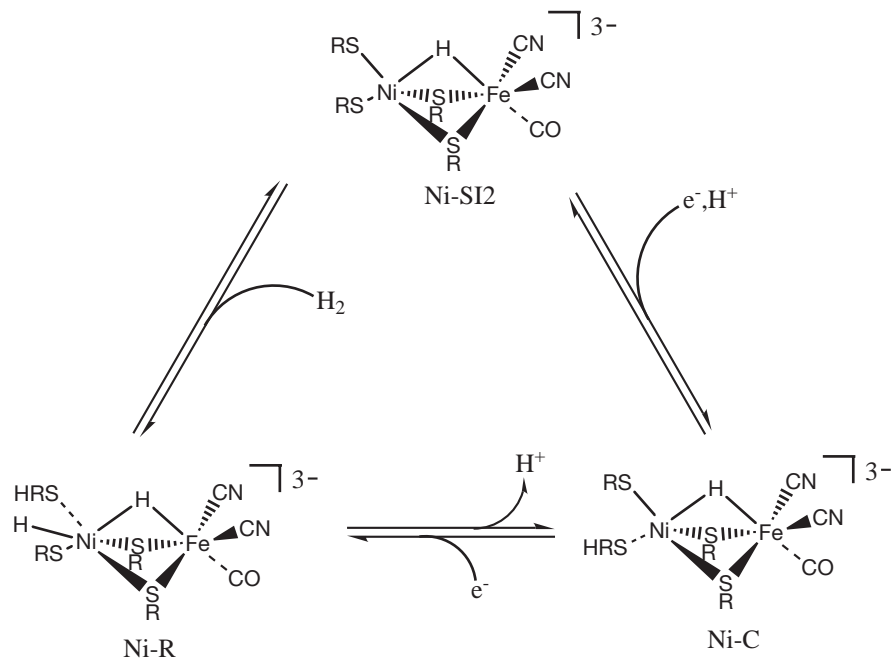


the catalytic cycle is the product of the  $\text{H}_2$  heterolytic splitting, which is found in the lower left corner in the cycle, and this structure is therefore assigned as Ni-C with a formal oxidation state of NiFe(II,III). It remains to assign Ni-R, which is EPR silent and for which there is also an X-ray structure [21]. This shows a quite short Ni-Fe distance, in line with the one found here for Ni-C, with an interatomic distance of 2.62 Å. This represents a shortening of 0.22 Å from the calculated value of the oxidized form, for which the original X-ray structure was determined [5] (which has a bridging oxygen derived ligand, see above). In the most recent paper [20] an assignment was made for Ni-R with a formal NiFe(III,III) oxidation state. Later investigations have shown that this structure is not stable with respect to rearrangement to a NiFe(II,II) complex (Wirstam M, to be published). At present, no structural assignment is therefore made for Ni-R, except that it should be similar to Ni-C with a bridging hydride but with an even number of electrons. Possibly, Ni-R could have the same structure as Ni-C with just an electron added. Finally, the reaction sequence was investigated by varying the number of protons on the cluster and thus varying the total charge, and it was found that the energetic results were rather insensitive to the total charge. Calculated frequencies were somewhat improved by using negatively charged clusters.

Amara et al. [22] have also used the same type of hybrid DFT method (B3LYP) to study the hydrogenase mechanism. As an interesting extension, a large part of the enzyme surrounding the complex was included using a combined quantum mechanics-molecular mechanics (QM-MM) model for the geometry optimizations and also for dynamical simulations. Both the geometry of the Ni-Fe complex and the calculated frequencies were found to be quite similar with and without the modeling of the protein environment. The effect of the protein is only about  $20 \text{ cm}^{-1}$  for the frequencies compared to an error of  $120 \text{ cm}^{-1}$  obtained for a free CO molecule using the same method. The protein effect on the bond distances is mostly in the range 0.00–0.05 Å, which is again in the same range as the uncertainty of the B3LYP method. For a few distances there are somewhat larger effects, but these involve quite energy-insensitive distances, such as for bridging ligands. It is therefore very unlikely that any difference in the assignment of intermediate states between this study and the one by Pavlov et al. [20] has anything to do with the additional modeling of the surrounding enzyme. Amara et al. proposed structures for some of the redox states by comparison of the optimized theoretical structures with available experimental data, as shown in Fig. 3.

The protonation states and oxidation states of the cluster were tested by comparing calculated spin den-

**Fig. 3** Catalytic cycle for Ni-Fe hydrogenase as proposed in [22]



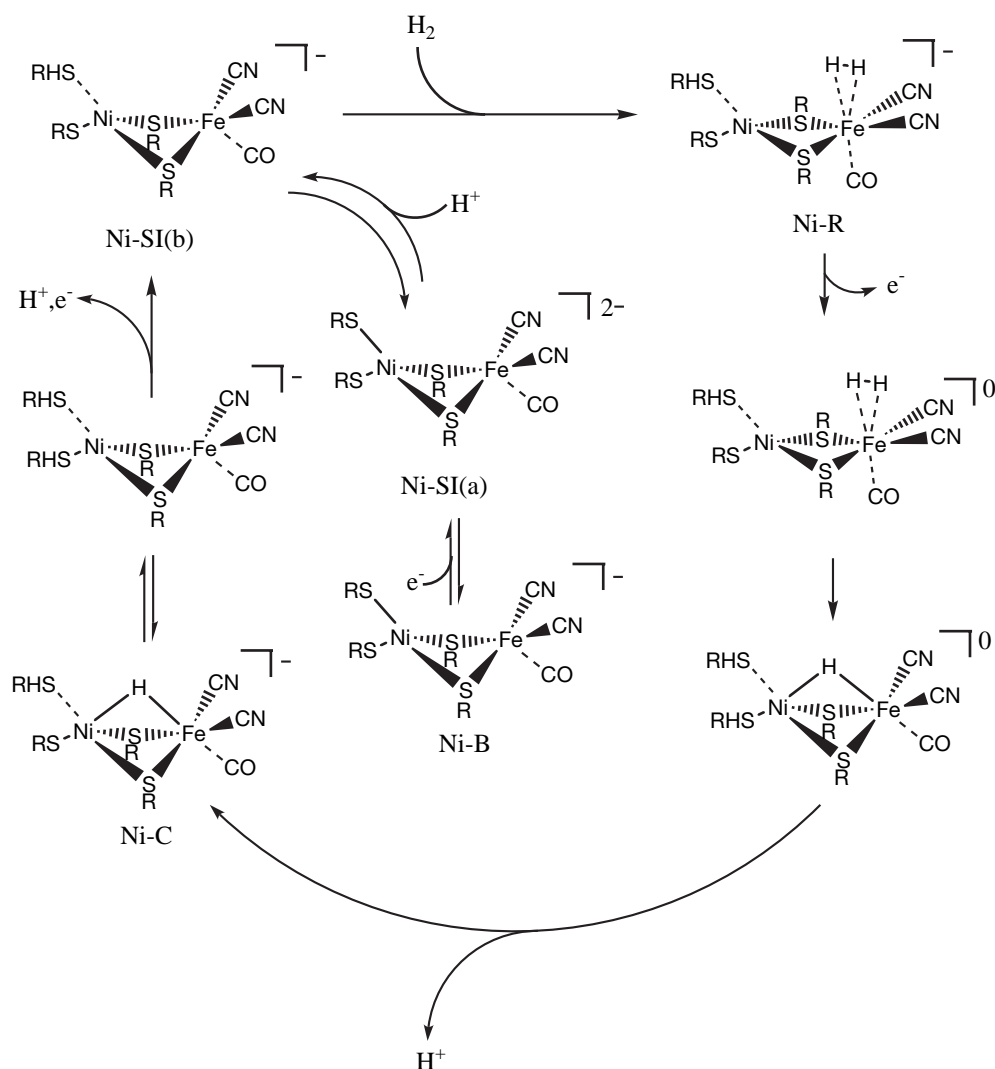
sities and vibrational frequencies with available EPR and IR spectroscopic data; surprisingly good agreement with experiment was obtained, in particular considering the inherent uncertainty of B3LYP frequencies for simple molecules like CO. The model of Amara et al. involves the same removal of the oxygen-derived group to generate the active catalyst as was discussed previously. Complexes with a charge of  $-3$  were used rather than the neutral clusters used in the study by Pavlov et al. The activation step was considered to occur at Ni rather than Fe. The argument for this is not that  $H_2$  is better bound on nickel but rather that the entering  $H_2$  is likely to encounter the Ni atom before the Fe atom. A significant difference from the model of Pavlov et al. is that a bridging hydride is suggested to be present throughout the catalytic cycle. This means that when  $H_2$  splits there is already a bridging hydride. This possibility for  $H_2$  splitting was investigated by Pavlov et al., but no low-energy path was found. It should be pointed out that this does not necessarily mean that no such pathway actually exists, since all possibilities cannot be tested. In the model proposed by Amara et al. in Fig. 3 the hydride produced from  $H_2$  ends up as a terminal ligand on nickel, whereas the proton becomes bound to one of the terminal cysteines on nickel. The reactant is identified as Ni-SI and the product as Ni-R. The  $H_2$  splitting is implied to occur on an EPR-silent Ni(II)Fe(II) complex, which is again in contrast to the study of Pavlov et al. where no EPR-silent complex was found to activate  $H_2$ . The principal difference between these studies is that in the study of Amara et al. the reaction sequence was derived based on a direct comparison to experimental data, such as IR and EPR data, while in the study of Pavlov et al. the

reaction pathway was derived by definitely locating the transition state quantum chemically. The third structure shown in Fig. 3 is obtained by removing one electron and one proton from Ni-R, leading to an Ni(I)Fe(II) complex, and this is identified with Ni-C. The alternative assignment of Ni-C as Ni(III)Fe(II) was also considered, since a hydrogen on one of the terminal cysteines was suggested to be formally regarded as a hydride rather than a proton.

Niu et al. [23] have also looked at the problem by the same hybrid DFT methods. They varied the cluster charge between  $-2$  and  $+1$  and used a procedure to assign the observed states which is different from both the above studies. They had noted that for a series of known  $LFe(CO)(CN)(L')^{n-}$  ( $L=Cp$  or  $Cp^*$ ,  $L'=CN, CO, CNCH_3$ ;  $n=0, 1, 2$ ) complexes the calculated C-O distances give a very accurate linear correlation with the logarithm of the experimental CO frequencies. The oxidation states and structures of the relevant intermediates were therefore determined by combining this calibration curve for the calculated CO bond distances of the active site model with the experimental enzyme IR frequencies. The proposed catalytic cycle is shown in Fig. 4.

They propose that  $H_2$  is activated at the Fe center by a Ni(III)Fe(II) complex to give a product species with a bridging hydride and a protonated cysteine residue. This is very similar to what was proposed by Pavlov et al. but different from the proposal by Amara et al. The only difference from the proposal by Pavlov et al. is that the protonated cysteine is a terminal ligand on nickel rather than a bridging (eventually terminal iron) ligand. The calculations by Pavlov et al. indicate that the energy difference between these structures is quite small. The H atoms are subsequently transferred to Ni

**Fig. 4** Catalytic cycle for Ni-Fe hydrogenase as proposed in [23]

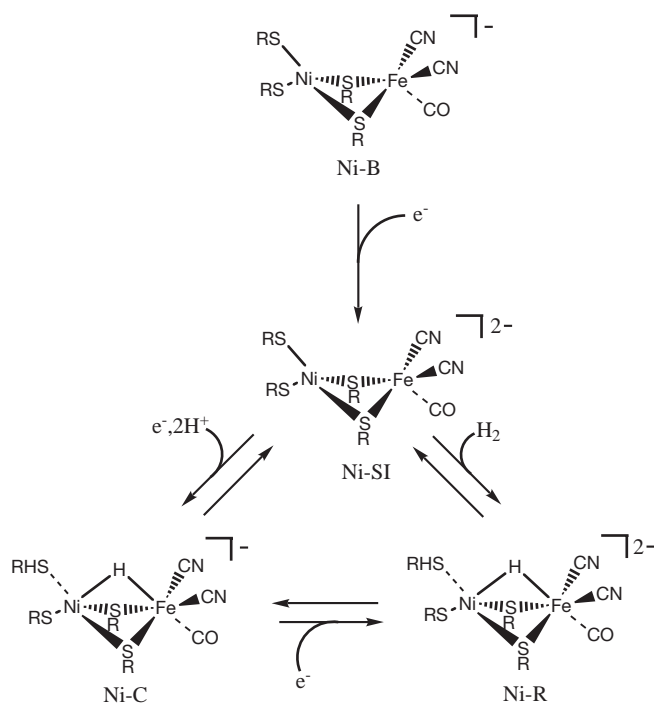


where they reside on protonated terminal Cys residues. Based on the calibration curve, Ni-B was proposed to be a Ni(III)Fe(II) species, Ni-SI(a) a Ni(II)Fe(II) species, and Ni-SI(b) was proposed to have a protonated terminal Cys. Ni-R was thought to be a Ni(II)Fe(II) cluster with H<sub>2</sub> bound at Fe, and Ni-C is considered as a Ni(III)Fe(II) species with an Fe-H-Ni bridge.

Finally, De Gioia et al. [24] have also studied the hydrogenase mechanism. In contrast to the above studies, they used the BLYP method which does not include Hartree-Fock exchange. The mechanism investigated was the one proposed by Dole et al. [25], for which they assigned observed intermediates as shown in Fig. 5. As in the scheme of Amara et al., they suggest H<sub>2</sub> splitting on an EPR-silent Ni(II)Fe(II) species, but they do not assume that a bridging hydride is present during the H<sub>2</sub>-splitting reaction. The assignments of the structures have many similarities to those made by Niu et al. and also to those made by Pavlov et al.; see further below. The main differences concern

the total charge of the complexes and thus the protonation states.

The proposals made in the four different studies may at first sight appear to be quite different. However, some of the differences in the assignments simply arise because the complexes have different overall charges. The charge of a complex in an enzyme depends both on the number of electrons (the oxidation states) and the number of protons in the complex. While the oxidation states are always highly significant, the number of protons present can often be a quite energy-insensitive property. In the enzyme there may well be many protonation states with nearly the same energy (within a few kcal/mol) that can be in equilibrium via proton transfers through the hydrogen bonding network. It is highly doubtful whether quantum chemical methods are accurate enough to always be able to determine precisely which one of these nearly degenerate configurations is the lowest one. Fortunately, in these near-degenerate situations, the



**Fig. 5** Catalytic cycle for Ni-Fe hydrogenase as proposed in [24]

exact protonation state is also one of the less interesting properties of these complexes.

If the above point concerning the protonation state is left out in the comparison of the different schemes, the similarities between the schemes now become striking. First, the structure of Ni-SI is now the same in the schemes of Pavlov et al., of Niu et al., and of De Gioia et al. It is an NiFe(II,II) complex without any hydride present. In the scheme of Pavlov et al., both terminal cysteines of nickel were selected as being protonated; in the scheme of Niu et al., one of them is protonated; and in the scheme of Gioia et al., none of them are protonated. These differences are minor from a quantum chemical modeling viewpoint. Ni-SI also occurs in the scheme of Amara et al., where it is suggested to be an NiFe(II,II) complex, but in this case there is also a bridging hydride present, which is a more significant difference.

The assignment of Ni-C also has many similarities in three of the above schemes. In these schemes, Ni-C is an NiFe(II,III) complex with one bridging hydride. Again there are differences in the protonation states, but this is less significant. On the other hand, in the scheme of Amara et al. the complex is suggested to be Ni(I)Fe(II) even though the possibility of Ni(III)Fe(II) is left open.

Ni-R is perhaps the most problematic state to assign, although all studies agree that it is an NiFe(II,II) complex. From the short distance found in the X-ray structure [21], it appears clear that there is a bridging hydride present. This agrees with three of

the assignments above. In the cycle of Amara et al., Ni-R also has a terminal hydride on nickel, while no such hydride is present in the study of De Gioia et al. In the version of the cycle proposed by Pavlov et al. discussed here, Ni-R is considered to be a reduced structure which lies outside the catalytic cycle.

The most interesting part of the catalytic cycle concerns the actual mechanism for the heterolytic cleavage of H<sub>2</sub>. A transition state structure was located by Pavlov et al. for an NiFe(II,III) complex and this was considered the most reliable result of that study. This conclusion was made since the possible alternatives all were found to have substantially higher energies. The proposed product of the splitting process places one hydrogen as a bridging hydride while the other hydrogen protonates a bridging cysteine. Almost the same mechanism was suggested by Niu et al., with the only difference being that a terminal cysteine is protonated instead. However, in contrast, in the studies by Amara et al. and by De Gioia et al., a mechanism where H<sub>2</sub> is split on a Ni(II)Fe(II) complex was suggested. In the mechanism of Amara et al. the splitting furthermore occurs for a complex where a bridging hydride is already present.

Quantum chemical methods provide a new and powerful technique with which to probe enzyme mechanisms at the molecular level. The broad measure of agreement between the proposals discussed here emphasizes the reliability of the method, once protonation state differences have been eliminated from consideration. The results give biophysical chemists definite predictions for testing and this should lead to much improved understanding in the future.

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