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<td>Citation</td>
<td>Proceedings of the Japan Academy, Series B. 89(1), p. 16-33</td>
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<td>Issue Date</td>
<td>2013-01-11</td>
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<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10297/7052">http://hdl.handle.net/10297/7052</a></td>
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<td>Version</td>
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Review

Studies on hydrogenase

By Tatsuhiko Yagi*1,† and Yoshiki Higuchi*2

(Communicated by Shigekazu Nagata, M.J.A.)

Abstract: Hydrogenases are microbial enzymes which catalyze uptake and production of H2. Hydrogenases are classified into 10 classes based on the electron carrier specificity, or into 3 families, [NiFe]-family (including [NiFeSe]-subfamily), [FeFe]-family and [Fe]-family, based on the metal composition of the active site. H2 is heterolytically cleaved on the enzyme (E) to produce EH2Hb, where Ha and Hb have different rate constants for exchange with the medium hydron. X-ray crystallography unveiled the three-dimensional structures of hydrogenases. The simplest [NiFe]-hydrogenase is a heterodimer, in which the large subunit bears the Ni-Fe center buried deep in the protein, and the small subunit bears iron-sulfur clusters, which mediate electron transfer between the Ni-Fe center and the protein surface. Some hydrogenases have additional subunit(s) for interaction with their electron carriers. Various redox states of the enzyme were characterized by EPR, FTIR, etc. Based on the kinetic, structural and spectroscopic studies, the catalytic mechanism of [NiFe]-hydrogenase was proposed to explain H2-uptake, H2-production and isotopic exchange reactions.

Keywords: hydrogenase, Ni-Fe center, cytochrome c3, isotope exchange, X-ray crystallography, catalytic cycle

Introduction

Hydrogenase1,2 was first described by Stephenson and Stickland as an enzyme which catalyzed activation of H2 to reduce electron acceptors such as O2, nitrate, fumarate, sulfate, or an artificial electron acceptor, methylene blue. Some hydrogenases catalyze the reverse reaction to produce H2 from electron donors such as methylviologen, benzylviologen or methylene blue, and are called reversible hydrogenases, whereas the enzymes unable to catalyze the H2-production are called uptake hydrogenases. Hydrogenases also catalyze isotopic exchange between dihydrogen and water, and conversion between para-H2 and ortho-H2.3–6 Hydrogenases are widely distributed in Bacteria and Archaea.7 In Eugarya, hydrogenases are found in unicellular algae8 and hydrogenosomes of unicellular Protozoa.9 Recent discovery10 of hydrogenosomes-like organelles in three species of animal phylum, Loricifera, living in sediments of the deep anoxic hypersaline L’Atalante basin, makes us expect for the presence of hydrogenase in animals.

Classification of hydrogenases

Classification based on the electron carrier specificity. During a few decades after the discovery1,2 of hydrogenase in 1931, the enzymatic activity had been demonstrated with artificial electron carriers such as methylviologen, benzylviologen or methylene blue, or by isotope exchange reactions, and the natural electron carriers had been out of concern. Mortenson et al.11 announced the discovery of ferredoxin (Fd), a small iron-sulfur (FeS) protein of low redox potential, from Clostridium pasteurianum, and demonstrated it to be a physiological electron carrier for clostridial hydrogenase (reaction 1). This enzyme is now registered as ferredoxin hydrogenase [Fd hydrogenase, EC 1.12.7.2] by IUBMB (International Union of Biochemistry and Molecular Biology, http://www.chem.qmul.ac.uk/iubmb/).

\[ H_2 + Fd = 2H^+ + \text{reduced Fd} \quad [1] \]
Purified hydrogenase from hydrogen bacteria, Desulfovibrio vulgaris Miyazaki (DvM), was shown to act on cytochrome c₃ (cyt-c₃) reversibly (reaction 2) but not on Fd, whereas clostridial hydrogenase was shown not to act on cyt-c₃. Hence the name cyt-c₃ hydrogenase [EC 1.12.2.1] was given to DvM hydrogenase. This established that hydrogenases from different origins may have different specificity to their electron carriers. Cyt-c₃ is a basic tetrahemoprotein of 14 kDa, and its four hemes are reduced successively according to the redox potentials (−280, −321, −325 and −356 mV).

2H₂ + ferricyt-c₃ (Fe³⁺)₄
= 4H⁺ + ferrocyt-c₃ (Fe²⁺)₄

[2]

Kinetic parameters for this reaction are: Kn[H₂] (pH 6.0)²⁰ = 16.5 μmol L⁻¹ (2.2 kPa H₂), Kₐ₅ (ferricyt-c₃, pH 7.0)²¹ = 2.6 μmol L⁻¹, Kn(ferrocyt-c₃, pH 6.0)²⁰ = 17 μmol L⁻¹, kₗ (for H₂ uptake, pH 7.0)²¹ = 49 s⁻¹, and kₗ (for H₂ evolution with saturating cyt-c₃ concentration, pH 6.0)²¹ = 336 s⁻¹.

Hydrogenases from other sulfate-reducing bacteria such as D. vulgaris Hildenborough (DvH),²³ D. gigas (Dg),²⁴ etc., act on their respective cyt-c₃s. Now, 10 kinds of hydrogenases with different electron carrier specificity are registered by IUBMB as shown in Table 1.

Some non-hydrogenase enzymes catalyze H₂ uptake or evolution. Nitrogenases [EC 1.18.6.1 and 1.19.6.1] produce H₂ as a by-product in the normal reaction to reduce N₂ to ammonia, and as a main product in the absence of N₂ at the expense of ATP,²⁷ carbon monoxide dehydrogenase/acetate-CoA synthase [EC 1.2.7.4 + 2.3.1.169] and pyruvate: Fd oxidoreductase [EC 1.2.7.1] reduce H⁺ to H₂ and, at much lower rate, oxidize H₂ to H⁺²⁸ and bacterial alkaline phosphatase [EC 3.1.3.1] catalyzes hydrolytic cleavage of the H–P bond of inorganic phosphate (HPO₄²⁻) to produce H₂ and phosphate (HPO₄²⁻).²⁹

Classification based on the structure of the active site. All hydrogenases except [EC 1.12.98.2]-enzymes can be classified into two families based on the structure of the active sites, i.e., [NiFe]-hydrogenases (active site: binuclear Ni-Fe center) and [FeFe]-hydrogenases (active site: binuclear Fe-Fe center). There is no correlation between the type of the active site and the electron carrier specificity. For
example, cyt-c₃ hydrogenases from DvM and Dg are of [NiFe]-type, whereas those from DvH and Desulfovibrio desulfuricans are of [FeFe]-type, and Fd hydrogenase from C. pasteurianum is of [FeFe]-type, whereas that from Methanosarcina is of [NiFe]-type. Some [NiFe]-enzymes have a selenocysteinyl (Sec) residue instead of one of the Cys residues supporting the Ni-Fe center, and are called [NiFeSe]-hydogenases.

The third family of hydrogenases, [Fe]-hydrogenases, consists of only [EC 1.12.98.2]-enzymes from methanogenic Archaea (Attention: in literature before 2002 when these enzymes had been believed to be metal-free enzymes, [Fe]-hydrogenases meant today’s [FeFe]-hydrogenases).

H-D exchange and para-H₂-ortho-H₂ conversion

The H-D exchange and the conversion between para-H₂ and ortho-H₂ are regarded as fundamental reactions of hydrogenases, because these reactions can be observed in the absence of any electron carrier. Rittenberg and Krassa used these reactions to elucidate the mechanism of action of hydrogenase of Proteus vulgaris, and proposed that the cleavage of H₂ by the enzyme (E) was heterolytic to form two unequal parts (reaction 3).

\[ H_2 + E \rightleftharpoons H^+ + E : H^- \]  \[ \text{[3]} \]

Here, H⁺ is readily exchangeable with the medium D⁺ in D₂O, but the enzyme-bound hydride (E : H⁻) is not. This conclusion was based on the observation that the ratio of H₂ and HD initially produced from H₂ over D₂O was determined to be nearly zero \( \left( \frac{v_{DD}}{v_{HD}} = 0 \right) \) from H₂/D₂O, \( \) and that the conversion of para-H₂ to ortho-H₂ did not occur over D₂O whereas the isotope exchange occurred \( (v_{o-o}/v_{o-x} = 0) \) from pH2/D2O, where \( v_{o-x} = v_{DD} + v_{DD} \). If both of the cleaved H species are partially exchangeable (i.e., partially unexchangeable) with the medium D⁺, either \( v_{DD}/v_{HD} \) or \( v_{o-o}/v_{o-x} \) must have not been zero. Tamiya and Miller reexamined these reactions and found that \( v_{DD}/v_{HD} \) from H₂/D₂O was dependent on the enzyme concentration, and was 0.19 at the enzyme concentration extrapolated to 0 for P. vulgaris enzyme. The \( v_{DD}/v_{HD} \) from H₂/D₂O was dependent on pH of the reaction medium, as well as on the bacterial source of the enzyme (e.g., 0.45 for C. pasteurianum enzyme at pH 7.0). The \( v_{o-o}/v_{o-x} \) from pH2/D2O was determined to be below the limit of detection (≤0.1) for enzymes from Proteus and Clostridium.

The initial \( v_{DD}/v_{HD} \) and \( v_{o-o}/v_{o-x} \) ratios from pH2/D2O, as well as the \( v_{DD}/v_{HD} \) from D₂/H₂O were reexamined by Yagi et al. when the DvM hydrogenase was purified (in retrospect, the purified enzyme contained about 70% of the active form, the remainder being inactive forms of the enzyme, wide infra), and simple and accurate assay of para-H₂ and ortho-H₂ became available. The exchange and the conversion reactions by the enzyme can be observed in the absence of added cyt-c₃, but much higher reaction rates were observed reproducibly in its presence (ferrocyt-c₃ was later found to influence the electronic structure of hydrogenase), hence the reactions were carried out in its presence. The \( v_{DD}/v_{HD} \) and \( v_{DD}/v_{HD} \) ratios were 0.20 and 0.40, respectively, at the enzyme concentration extrapolated to 0, but were 1.5 and 3.6, respectively, when the enzyme concentration was extremely high, because the dihydrogen species liberated from the enzyme had additional chances to be caught by the enzyme for additional exchange reactions (cage effect) before release to the gas phase. The \( v_{o-o}/v_{o-x} \) ratio was higher at lower enzyme concentration, and were as high as 0.7 at the enzyme concentration extrapolated to 0. These experimental results were successfully explained by the mechanism schematically represented in Fig. 1. The kinetic parameters which are compatible with these experimental data were calculated, and given in the figure. This scheme conforms to the heterolytic cleavage of the enzyme-bound H₂, i.e., one of the enzyme-bound H₂ has the rate constant \( (k_0) \) for the isotope exchange reaction 10 times as that \( (k_0) \) of the other, but the mode of heterolysis is not as simple as what was initially suggested as in reaction 3. Any structural model proposed for the enzyme-bound H₂ must conform to the scheme shown in Fig. 1.

The catalytic mechanism of hydrogenase proposed in Fig 1 suggests that water does not participate in para-H₂-ortho-H₂ conversion. In fact, dry hydrogenase was proved to catalyze the conversion reaction at a reaction rate \( (k = 1.3 s^{-1}) \) about 1/340 of that in aqueous reaction in the absence of cyt-c₃. The isotopic exchange reaction between H₂ and D₂ to produce HD was also observed with dried hydrogenase–cyt-c₃ mixture with a reaction rate 20% that of the conversion reaction. This indicates that the covalent bond of H₂ is split on the enzyme molecule and the bound H (Hα or Hβ, or both) is exchangeable with hydrons (H⁺ or D⁺) on the protein molecule. When a mixture of hydrogenase and excess cyt-c₃ (400 times in molar base, i.e., 63 times by
Kinetic parameters for pH2/D2O

\[ \frac{v_{HH}}{v_{HD}} = 0.40 \text{ from D2/H2O} \]

The rate constants, \( k_{1} \) and \( k_{2} \), are those for the dissolution and evolution of H2 and from D2/H2O, and are dependent on the shape of the reaction vessel, shaking speed, etc., but not on the enzyme concentration. Dihydrogen (HH) dissolved in D2/H2O is bound to the enzyme (E) with the rate constant \( k_{2} \), and is dissociated from the enzyme-H2 (EHH) with \( k_{-2} \). Heterolytic cleavage of H2 on E makes two H atoms to bind at positions a and b. The rate constant for exchange with medium D2 at position a to form EDaHb is \( k_{a} \) and that at position b to form EDaDb is \( k_{b} \) (the concentration of D2 in D2/H2O is included in \( k_{a} \) and \( k_{b} \)). EDaHb and EHaDb are dissociated to produce HD at the rate constant \( f_{2}k_{2} \), where \( f_{2} \) is the factor for the isotope effect. The enzyme-bound HDs (EDaHb + EHaDb) exchange with medium D2 at the rate constants, \( k_{a} \) and \( k_{b} \), respectively, to produce EDaDb, from which the final product D2 is dissociated. The isotopic exchange reaction between D2 and H2O (D2/H2O) to produce HD and H2 can be figured out similarly. The results of the isotopic exchange and the conversion reactions shown in this figure are compatible with the calculated kinetic parameters.

That is, when 100 parts of para-H2 are bound to the enzyme according to this mechanism, the resulting EDaHb liberates 41.1 (= 100k2/k1 + k2 + k1) parts of normal H2 and is converted to 53.5 parts of EDaHb, and 5.4 parts of EDaDb. The 53.5 parts of EDaHb liberate 46.8 (= 53.5f2k2/k1 + k2 + k1) parts of HD, and the 5.4 parts of EDaDb liberate 2.2 (= 5.4f2k2/k1 + k2 + k1) parts of HD, and the remainder (100 – 41.1 – 46.8 – 2.2 = 9.9 parts) is converted to EDaDb to produce D2. The dihydrogen species (H2, HD and D2) liberated to the gas phase without being trapped by the enzyme in a solution at extrapolated 0 enzyme concentration, are composed of 41.1 parts normal H2, 49.0 (= 46.8 + 2.2) parts HD and 9.9 parts D2. Thus the calculated \( \frac{v_{DD}}{v_{HD}} \) (9.9/49.0 = 0.20) and \( \frac{v_{DD}}{v_{ex}} \) (41.1/49.0 + 9.9 = 0.70) are in good agreement with those observed experimentally.

Initial ratios, observed at enzyme concentration extrapolated to 0. \( \frac{v_{DD}}{v_{HD}} = 0.20 \), \( \frac{v_{HH}}{v_{ex}} = 0.70 \) from pH2/D2O

\( \frac{v_{HH}}{v_{ex}} = 0.40 \) from D2/H2O

Kinetic parameters for pH2/D2O

\[ \begin{align*}
&k_{1} = 5.2 \times 10^{-8} \text{ Pa}^{-1} \\
&k_{2} = 4.3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1} \\
&k_{3} = 6.9 \times 10^{11} \text{ mol}^{-1} \text{ s}^{-1} \\
&k_{5} = 430 \text{ s}^{-1} \\
&k_{-5} = 0.9 \\
&k_{b} = 56 \text{ s}^{-1}
\end{align*} \]

Kinetic parameters for D2/H2O

\[ \begin{align*}
&k_{1} = 5.8 \times 10^{10} \text{ Pa}^{-1} \\
&k_{2} = 9.5 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1} \\
&k_{3} = 4600 \text{ s}^{-1} \\
&k_{5} = 1.1 \\
&k_{-5} = 460 \text{ s}^{-1}
\end{align*} \]

Fig. 1. Diagrammatic representation of the exchange and conversion reactions of para-H2 over D2O (pH2/D2O) catalyzed by hydrogenase. The reaction was carried out under the H2 pressure of 20 kPa at 303 K. The rate constants, \( k_{1}, k_{2} \) and \( k_{3} \), are for the dissolution and evolution of H2 and from D2/H2O, and are dependent on the shape of the reaction vessel, shaking speed, etc., but not on the enzyme concentration. Dihydrogen (HH) dissolved in D2/H2O is bound to the enzyme (E) with the rate constant \( k_{2} \), and is dissociated from the enzyme-H2 (EHH) with \( k_{-2} \). Heterolytic cleavage of H2 on E makes two H atoms to bind at positions a and b. The rate constant for exchange with medium D2 at position a to form EDaHb is \( k_{a} \) and that at position b to form EDaDb is \( k_{b} \) (the concentration of D2 in D2/H2O is included in \( k_{a} \) and \( k_{b} \)). EDaHb and EHaDb are dissociated to produce HD at the rate constant \( f_{2}k_{2} \), where \( f_{2} \) is the factor for the isotope effect. The enzyme-bound HDs (EDaHb + EHaDb) exchange with medium D2 at the rate constants, \( k_{a} \) and \( k_{b} \), respectively, to produce EDaDb, from which the final product D2 is dissociated. The isotopic exchange reaction between D2 and H2O (D2/H2O) to produce HD and H2 can be figured out similarly. The results of the isotopic exchange and the conversion reactions shown in this figure are compatible with the calculated kinetic parameters. That is, when 100 parts of para-H2 are bound to the enzyme according to this mechanism, the resulting EDaHb liberates 41.1 (= 100k2/k1 + k2 + k1) parts of normal H2 and is converted to 53.5 parts of EDaHb, and 5.4 parts of EDaDb. The 53.5 parts of EDaHb liberate 46.8 (= 53.5f2k2/k1 + k2 + k1) parts of HD, and the 5.4 parts of EDaDb liberate 2.2 (= 5.4f2k2/k1 + k2 + k1) parts of HD, and the remainder (100 – 41.1 – 46.8 – 2.2 = 9.9 parts) is converted to EDaDb to produce D2. The dihydrogen species (H2, HD and D2) liberated to the gas phase without being trapped by the enzyme in a solution at extrapolated 0 enzyme concentration, are composed of 41.1 parts normal H2, 49.0 (= 46.8 + 2.2) parts HD and 9.9 parts D2. Thus the calculated \( \frac{v_{DD}}{v_{HD}} \) (9.9/49.0 = 0.20) and \( \frac{v_{DD}}{v_{ex}} \) (41.1/49.0 + 9.9 = 0.70) are in good agreement with those observed experimentally.

Development of continuous mass-spectroscopic monitoring system \(^{42}\) of isotopic dihydrogens greatly simplified the exchange studies. Table 2 summarizes the results of isotope exchange and para-H2-ortho-H2 conversion reactions reported in literature. The \( \frac{v_{DD}}{v_{HD}} \) (from D2/H2O) or \( \frac{v_{HH}}{v_{ex}} \) (from D2/H2O) varies significantly among hydrogenases from different origins. Extremely higher \( \frac{v_{DD}}{v_{HD}} \) or \( \frac{v_{HH}}{v_{ex}} \) ratios observed with membrane particles, organelles or cells must have been due to the cage effect, \(^{43}\) because enzymes are expected to be locally concentrated, or free diffusion of dihydrogen species are limited there.

For the purified [NiFe]- and [FeFe]-enzymes, the \( \frac{v_{DD}}{v_{HD}} \) and \( \frac{v_{HH}}{v_{ex}} \) ratios are 0.2–0.4 with few exceptions, and the \( \frac{v_{HH}}{v_{ex}} \) ratios are 0.3–0.6, with a notable exception for [NiFeSe]-enzyme. These data may be considered proof for the heterolytic cleavage of dihydrogen, but strictly speaking, \( k_{a}/k_{b} \) ratio can be calculated only when \( v_{DD}/v_{ex} \) and \( v_{HH}/v_{ex} \) ratios (from pH2/D2O) were measured simultaneously. For example, \( v_{DD}/v_{ex} \) ratio could be 0.2 for H2 homolysis (\( k_{a} = k_{b} = 0.18k_{-1} \) in Fig. 1) if \( v_{HH}/v_{ex} \) were determined to be 2.8.

Higher \( \frac{v_{DD}}{v_{HD}} \) or \( \frac{v_{HH}}{v_{ex}} \) ratios were observed for Ni-rubredoxin (rubredoxin with its tetrahydro complexes Fe\(^{2+}\)/\(^{3+}\) ion substituted with Ni\(^{2+}\) ion) \(^{44}\) and a binuclear NiRu complex (Ni\(^{2+}\)(μ-SR)2(μ-H)Ru\(^{3+}\)). \(^{45}\) The mode of H2 splitting by Ni-rubredoxin, which had been prepared before the structure of the Ni-Fe center was elucidated, could either be homolytic (e.g., \( k_{a} = k_{b} = 1.3k_{-1} \) in Fig. 1) or heterolytic (e.g., \( k_{a} = 3k_{b} = 3k_{-1} \) depending on the \( v_{HH}/v_{ex} \) ratio which had not been determined. This complex also catalyzed production of H2 from methylviologen cation radical. As the catalytic activities of Ni-rubredoxins depended on the sources of rubredoxin, \(^{51}\) the protein part must have been important for the catalytic function. The mode of H2 splitting by Ni-Ru complex, \(^{52}\) which had been designed to mimic the Ni-Fe center of [NiFe]-hydrogenase, could also be either homolytic (e.g., \( k_{a} = k_{b} = 3.1k_{-1} \) or heterolytic (e.g., \( k_{a} = 2k_{b} = 5k_{-1} \)). This complex catalyzed reduction of ketone to alcohol with H2.
Amino acid sequence and subunit composition of hydrogenases

The amino acid sequence of DvH [FeFe]-hydrogenase was first reported by Voordouw.31) (Database accession: P07598 and P07603 for the heterodimer). The sequences of Dg [NiFe]-hydrogenase (Fig. 2), Desulfomicrobium baculatum [NiFeSe]-enzyme (Fig. 2), C. pasteurianum [FeFe]-enzyme (P29166) and Methanocaldoccus jannaschii [Fe]-enzyme (Q58194) were then reported. The sequences of [NiFe]-family, [FeFe]-family and [Fe]-family enzymes are phylogenetically unrelated. It is intriguing to note that, whereas majority of hydrogenases expressed in Bacteria and Archaea are of [NiFe]-type,7) only [FeFe]-enzymes are expressed in Eucarya.

Every [NiFe]-hydrogenase is composed of a catalytically active core heterodimer, with or without additional subunit(s). Cyt-c3 hydrogenases from sulfate reducing bacteria (Desulfovibrio, Desulfomicrobium, etc.) have no additional subunit,35),53),54) and are the simplest among the [NiFe]-family. Quinone-reducing hydrogenase7),55) and methanophenazine hydrogenase7),56),57) have membrane-bound dihemic cytochrome b as the third subunit to interact with the electron carrier, menaquinone and methanophenazine, respectively. F420-reducing hydrogenase from methanogenic archaeon is an oligomer of trimers consisted of the core heterodimer and another subunit.58) NAD\textsuperscript{D}-reducing hydrogenase from hydrogen bacterium, Ralstonia eutropha H16 has four more subunits, two of which constitute diaphorase to mediate electron transfer between the core heterodimer and NAD\textsuperscript{D}/NADH.59) The sequence alignments of the core heterodimers of some [NiFe]-hydrogenases (Fig. 2) indicate that they are homol-
ogous regardless whether the enzymes are of archaeal (No. 6) or bacterial origins, or they are cytoplasmic (No. 7) or periplasmic. [FeFe]-hydrogenase may be a monomer (Fd hydrogenase from *C. pasteurianum*,33),63) *Megasphaera elsdenii*,64) etc.), a heterodimer (e.g., *DvH cyt-c*), a heterotrimer (e.g., bifurcating hydrogenase65)), or a heterotetramer (e.g., *D. fructosovorans* NADP*-reducing hydrogenase*66)). [Fe]-hydrogenase is a homodimer composed of only 1 gene product.67),68)

**Three-dimensional structure of hydrogenases**

The X-ray structure of Dg [NiFe]-hydrogenase was elucidated by French group,69),70) and then that of DvM [NiFe]-enzyme (Fig. 3a), by the authors.71) The structures of [FeFe]-enzymes from *C. pasteurianum*72) and *D. desulfuricans*,32) [NiFeSe]-...
enzyme from *D. baculatum*,\(^{73}\) and [Fe]-enzyme from *M. jannaschii*\(^{88}\) were successively determined.

**Standard [NiFe]-hydrogenase.** Hydrogenases from *DvM* and *Dg* are very similar in the main-chain folding, reflecting their sequence homology (67% identical, Fig. 2). The Ni-Fe center is held by 4 Cys residues of the large subunit, and is buried deep in the center of the protein.\(^{69}-71\) The Fe atom of the Ni-Fe center is coordinated by 3 diatomic ligands (Fig. 3b).\(^{74}\) An additional metal center, Mg\(^{2+}\)-center, also held by the large subunit was revealed by 1.8 Å resolution crystallography.\(^{71}\) Three FeS clusters, i.e., proximal and distal [4Fe-4S] clusters and mesial [3Fe-4S] cluster from the Ni-Fe center are held by the small subunit, and align linearly. The binding site for cyt-\(c\)_\(3\) was suggested to locate near the distal [4Fe-4S] cluster (Fig. 3a).\(^{21}\) H\(_2\) is supposed to access the Ni-Fe center through hydrophobic...
channels connecting the Ni-Fe center to the protein surface.\textsuperscript{75}–\textsuperscript{77}

The structures of Ni-Fe centers from Dg and DvM enzymes are similar, but subtly differ from each other. The bridging ligand (purple sphere in Fig. 3b) between Ni and Fe was assigned as oxygen species (OH or O\textsuperscript{2}\textsuperscript{-}) for Dg enzyme,\textsuperscript{70} but as sulfur species (S\textsuperscript{-} or SH) for DvM enzyme.\textsuperscript{71} This might reflect the fact that DvM enzyme had been purified under strict anaerobiosis and was active as isolated, whereas Dg enzyme was prepared under aerobiosis as an inactive unready state, which could be activated only after prolonged incubation under H\textsubscript{2}.\textsuperscript{78} It was later established that the bridging ligand of the unready and ready forms were hydroperoxide and hydroxide species for \textit{D. fructosovorans} enzyme,\textsuperscript{78} and disappeared upon reductive activation.\textsuperscript{79} When DvM enzyme was incubated under H\textsubscript{2} in the presence of cyt-c\textsubscript{3}, H\textsubscript{2}S was liberated\textsuperscript{80} and the enzyme lost the bridging sulfur species at the Ni-Fe center.\textsuperscript{81} DvM enzyme, on oxidation, was shown to have OH\textsuperscript{-} as the bridging ligand by EPR and electron-nuclear double resonance spectroscopy applied to single crystals of the enzyme,\textsuperscript{82} and the bridging ligands of the Ni-A and Ni-B forms of DvM enzyme were suggested to be peroxy (or hydroperoxo) and oxo species, respectively.\textsuperscript{83}

Atomic species of three diatomic ligands to Fe are enigmatic. These were assigned as 1 CO and 2 CN\textsuperscript{-} for Dg enzyme,\textsuperscript{74} as well as for similar [NiFe]-enzyme from \textit{Chromatium vinosum},\textsuperscript{84} whereas the electron density of one of three ligands (L1 of Fig. 3b) of DvM enzyme was too big to be modeled as CN\textsuperscript{-} or CO, and had to be assigned as SO.\textsuperscript{71} The presence of a rather bizarre SO ligand had precedents in synthetic coordination compounds,\textsuperscript{85} and SO (m/z = 48) began to be released from the native DvM enzyme by mass spectrometry at about 400 K.\textsuperscript{86} far below the decomposition temperature of covalent bonds in proteins and FeS clusters. However, later enzyme lots prepared from different batches of the bacterium had the diatomic ligands similar in size to those in Dg enzyme.\textsuperscript{87} Possibility of erroneous assignment as SO in the earlier study\textsuperscript{71} is improbable unless other source of SO is specified, because SO was detected by the technique\textsuperscript{86} different from X-ray crystallography. Subtle difference in culturing bacterium might have affected the diatomic ligand composition, but the way how the culturing conditions affect the ligand composition has not yet been figured out. Some residual electron densities (not fully occupied monatomic species, O or S) were observed near the S atoms of some Cys residues (Cys84 and Cys546 for DvM enzyme).\textsuperscript{75,83,87}

**O\textsubscript{2}-tolerant [NiFe]-hydrogenase.** Membrane-bound [NiFe]-hydrogenase from \textit{Hydrogenovibrio marinus} is extremely thermophilic and functions in the presence of O\textsubscript{2}.\textsuperscript{88} the properties unique among hydrogenases. The Ni-Fe center of this enzyme is similar to that of the standard [NiFe]-enzyme from DvM or Dg (Fig. 3b), but the proximal FeS cluster is not of [4Fe-4S]-type, but of [4Fe-3S]-type (Fig. 4).\textsuperscript{69} The Gly residue in the conserved CTC*CS (G asterisked to be highlighted) segment at the binding site to the proximal [4Fe-4S] cluster in the standard [NiFe]-enzyme is substituted by Cys65, and the 2nd Gly residue in the conserved GG*VQAA segment also at the binding site to the same [4Fe-4S] cluster in the standard enzyme is substituted by Cys166 residue (CTC*CS and GC*VQAA segments of the small subunit in Fig. 2). Instead of the missing S\textsuperscript{2-} ion which would have coordinated to Fe1, Fe2 and Fe4 of the standard enzyme, Cys65-thiolate coordinates to Fe1 and Fe2, and Cys166-thiolate coordinates to Fe4, to make the proximal [4Fe-3S] cluster in the reduced form of \textit{H. marinus} enzyme (Fig. 4a). In the standard [4Fe-4S] clusters, each Fe ion in [4Fe-4S] clusters is held by a single Cys-thiolate (Fig. 4c), but in the [4Fe-3S] cluster of \textit{H. marinus} enzyme, each of three Fe ions (Fe1, Fe2 and Fe4) is doubly coordinated by 2 Cys-thiolates, therefore the [4Fe-3S] cluster is more stably embraced by the protein than the standard [4Fe-4S] clusters. On oxidation of the enzyme, the vertical Fe2–S3 bond of the [4Fe-3S] cluster in Fig. 4a is cleaved, and Fe2 becomes coordinated by amide N of Cys66 backbone, resulting in significant conformational change of the cluster (Fig. 4b), without being thrown out from the protein. The cleavage of the Fe2–S3 bond makes the highly conjugated system of the [4Fe-3S] cluster to loosely conjugated 2 subparts, to enable the [4Fe-3S] cluster to conduct two-electron redox change to reduce the oxygen species introduced to the Ni-Fe center during the catalytic cycle in the presence of H\textsubscript{2} and O\textsubscript{2}. Another membrane-bound O\textsubscript{2}-tolerant [NiFe]-hydrogenase from \textit{R. eutropha}\textsuperscript{89} (P31892, not the soluble enzyme 7 in Fig. 2) has a similar [4Fe-3S] cluster held by the same CTC*CS and GC*VQAA sequences\textsuperscript{90} as those of \textit{H. marinus} hydrogenase, and the mutation of both Cys\textsuperscript{8} residues to Gly converted the enzyme to a standard O\textsubscript{2}- sensitive hydrogenase.\textsuperscript{89}

**[NiFeSe]-hydrogenase.** The structure of [NiFeSe]-hydrogenase from \textit{D. baculatum} in the
reduced form,\textsuperscript{73} is similar to the standard [NiFe]-enzymes from DvM and Dg, except that one of Cys residues coordinated to the Ni atom is replaced by Sec (U493 in Fig. 2), the monatomic center has Fe instead of Mg in the large subunit, and a [4Fe-4S] cluster instead of the mesial [3Fe-4S] cluster in the small subunit. The three diatomic ligands to Fe are 1 CO and 2 CN as in the case of the standard enzymes, but the bridging site between Ni and Fe is vacant as isolated. Intriguingly, at position 6.7 Å from the Ni-Fe center, H$_2$S is retained, which might have been liberated from the Ni-Fe center, but these authors\textsuperscript{73} argued against the ex-presence of sulfur species at the Ni-Fe bridging site. The [NiFeSe]-hydrogenase as isolated from DvH also has a vacant bridging site between Ni and Fe.\textsuperscript{76} [NiFeSe]-enzymes are known to be O$_2$-tolerant, but the mechanism of O$_2$-tolerance was suggested to be due to prevention of

Fig. 3. The X-ray structure [PDBid 1WUJ] of [NiFe]-hydrogenase, 89 kDa protein, from DvM.\textsuperscript{83} (a) The overall structure. The peptide main chains of the heterodimeric protein are represented by semitransparent ribbon form to show the Ni-Fe and Mg centers and the FeS clusters, which are buried in the protein. The binding site\textsuperscript{21} for its electron carrier, cyt-c$_3$, is indicated. (b) The binuclear Ni-Fe center. The residue numbers are of DvM enzyme. Subtract 16 for the residue numbers of Dg enzyme. L1, L2 and L3 are diatomic ligands to Fe, and S/O (S or O) is the ligand bridging Ni and Fe. An oxygen atom (O) modeled on the S atom of Cys546 (which is in sulfinic form of Cys) was not observed in some preparations [e.g., PDBids 1H2A and 2FRV].

Fig. 4. Comparison of the proximal FeS clusters of the O$_2$-tolerant and the standard [NiFe]-hydrogenases. (a) Reduced and (b) oxidized forms of the [4Fe-3S] cluster of the O$_2$-tolerant [NiFe]-hydrogenase from \textit{H. marinus}. (c) The [4Fe-4S] cluster of the standard [NiFe]-hydrogenase from DvM. Instead of one S$_2$ ion (S2) of the [4Fe-4S] cluster which coordinates to Fe1, Fe2 and Fe4, Cys65 coordinates to Fe1 and Fe2, and Cys166, to Fe4 in the [4Fe-3S] cluster.
O₂ accession through H₂ channels by Sec-selenol and monatomic extra atom near the bridging Cys residue to Ni and Fe. In addition, its proximal [4Fe-4S] cluster has features different from those of the standard [NiFe]-enzymes.

**[FeFe]-hydrogenase.** Heterodimeric [FeFe]-hydrogenase from *D. desulfuricans* differs from the [NiFe]-enzyme in protein folding. The active site called H-cluster is composed of a binuclear Fe-Fe center and a [4Fe-4S] cluster, both of which are held by a Cys thiolate of the large subunit, and is buried deep in the center of the protein. Instead of 2 Cys residues bridging Ni and Fe atoms of the Ni-Fe center in the [NiFe]-enzyme, two dithiolate groups of 2-(aza or oxa)propane-1,3-dithiol are bridging two Fe atoms of the Fe-Fe center (Fig. 5a). Two other [4Fe-4S] clusters are also held by the large subunit. In spite of these differences from the [NiFe]-enzyme, the Fe atoms at the Fe-Fe center coordinate diatomic CO and CN⁻ ligands as in the Ni-Fe center of the [NiFe]-enzyme. The monomeric [FeFe]-enzyme from *C. pasteurianum* has a similar H-cluster as that of *D. desulfuricans* enzyme, but differs in having extra [4Fe-4S] and [2Fe-2S] clusters.

**[Fe]-hydrogenase.** The active site of [Fe]-hydrogenase locates on the protein surface, has ligands of 6-carboxymethyl-4-(5′-guanylyloxy)-3,5-dimethylpyridin-2-ol and 2 CO, and is supported by Cys-thiolate of the protein (Fig. 5b). Unlike the hydrogenases of the other families, both H₂ and the electron mediator, 5,10-methylenetetrahydromethanopterin displace H₂O with its re-face of methenyl-C facing to Fe, then H₂ is inserted between Fe and the acceptor.

**Catalytic mechanism of [NiFe]-hydrogenase**

**Activation of the enzyme.** Dg [NiFe]-hydrogenase as purified aerobically is in the inactive unready state, which is activated only after prolonged incubation under H₂. The activated enzyme can be oxidized anaerobically to the ready state, which is inactive but readily activated on reduction. The unready, ready and active states were correlated to the EPR signals dubbed Ni-A (g = 2.31, 2.26, 2.02), Ni-B (g = 2.33, 2.16, 2.02) and Ni-C (g = 2.19, 2.14, 2.02), respectively. The interfering signal of the mesial [3Fe-4S] cluster can be quenched by measuring EPR at temperature above 50 K. The Fe atom is diamagnetic at any redox state of the enzyme. FTIR spectroscopy, in combination with EPR and electrochemistry distinguished different states of the enzyme, including the EPR silent states. The activation/inactivation pathways of the enzyme in various redox states are illustrated in Fig. 6 (upper 2 rows), where direct oxygenation of Ni-B to Ni-A form has not been observed with Dg enzyme. Activation of Ni-B₁₉₄₆ (see the legend to Fig. 6 for the subscript) to Ni-SI₁₉₁₄ form may not proceed in one step as indicated, but Ni-B₁₉₄₆ may be reduced (Dₑ < 1) followed by the removal of the bridging ligand (−OH⁻).

DvM [NiFe]-hydrogenase is active or readily activated as isolated. Some preparations had only an EPR signal at g = 2.017 of [3Fe-4S] cluster, whereas others had additional signals (g = 2.32, 2.23, 2.16, 2.01) of combined Ni-A and Ni-B forms. It was later recognized that the accession of air to the enzyme during purification was not carefully con-
trolled at that time, so that some preparations contained detectable amounts of Ni-A ($g = 2.32, 2.24, 2.01$) and Ni-B ($g = 2.33, 2.16, 2.01$) forms, but others contained hardly detectable amounts of Ni-A and Ni-B forms. The enzyme purified under aerobiosis contained 70% Ni-B form, and behaved as an active enzyme in the assay system, in which the enzyme was reduced chemically with dithionite or electrochemically to start the reaction. The redox titration of the enzyme having EPR Ni-signals with sodium dithionite led to successive disappearance of Ni-B and Ni-A signals at midpoint potentials of $-230$ and $-310$ mV, respectively. Ni-C signal ($g = 2.196, 2.144$) appeared at potential $-370$ mV, reached a maximum at about $-400$ mV, and disappeared at $-430$ mV. Ni-B form (having oxo or hydroxo species as the bridging ligand between Ni and Fe) can be converted to Ni-A form (having peroxo or hydroperoxo species as the bridging ligand) by treatment with Na$_2$S to form...
Ni-B’ ($g = 2.29, 2.14, 2.00$), followed by the addition of O$_2$. Ni-Fe-supporting Cys residues$^{33,87}$ might be related to some behaviors of the enzyme (e.g., evolution of H$_2$S on activation, activation/inactivation of various states of the enzyme, etc.), but they are disregarded in the following discussion, because we do not know the real structure at work in vivo.

It is noteworthy that in spite of differences in the bridging ligand between Ni and Fe, both of DvM and Dg enzymes, on activation, behave similarly in redox processes.$^{30}$

Catalytic cycle. Based on the kinetic, structural, and spectroscopic studies described in the preceding sections, the catalytic cycle of [NiFe]-hydrogenase was proposed to explain H$_2$-uptake, H$_2$-production and isotopic exchange reactions, as shown in the lower part of Fig. 6.$^{77}$ Only the 3 structures present in the lower pH range (i.e., protonated forms shown in the shaded boxes in the inner circuit of the catalytic cycle) were proved to be catalytically active.$^{74}$ We think, however, that both protonated and deprotonated Ni-SI$(-)$ forms have catalytic activity and the inner and outer circuits of the catalytic cycle are operating, because hydrogenase is active in a wide pH range, and the pH optimum for H$_2$ evolution (pH$_{opt}$) is lower than the pK$_a$ between Ni-SI$(-)$$_{1914}$ and Ni-SI$(-)$$_{1934}$, that of H$_2$ uptake (pH$_{opt}$) is higher than that pK$_a$, and that of the isotopic exchange is between pH$_{opt}$$^+$ and pH$_{opt}$$^-$.  

H$_2$ diffused from the protein surface through hydrophobic tunnels$^{75-77}$ is suggested to bind at the 6th ligand site of Ni of Ni-SI forms of the enzyme, because gaseous CO, the competitive inhibitor,$^{15}$ was found to bind at this site,$^{57}$ but see the legend to Fig. 6 for other possibilities. Inactivity of Ni-A form can be explained by the fact that the outer atom of the diatomic bridge of this form occupies the same position as that of CO in the CO-bound enzyme to prevent H$_2$ from binding to Ni. Heterolytic cleavage of the bound H$_2$ produces Ni-R form, in which the hydride is believed to bind at the Ni-Fe bridging site,$^{26,98-100}$ and H$^+$ on the S atom of Cys* (Cys* is Cys546 for DvM enzyme).$^{93}$ In the H$_2$-uptake reaction to reduce ferriyect-c$_3$, the outer circuit starting from NiSI$_{1914}$ form operating clockwise to produce (Ni-R) form (deprotonated form of NiR$_{1914}$, not confirmed by spectroscopy) must be predominant, because pH$_{opt}$$^+$ lies above the pK$_a$. The resulting (Ni-R) form, then transfers an electron to ferricyct-c$_3$ through the proximal, mesial and distal FeS clusters, from which the edge-to-edge distant to the nearest heme of the bound ferricyct-c$_3$ is only 6.4 Å, with π-orbital rings of His238 and Phe247 located inbetween.$^{21}$ The resulting (Ni-C) form transfers the 2nd electron to ferricyct-c$_3$ to regenerate Ni-SI$_{1914}$ to complete the cycle. The H$^+$ produced can be released to the medium through a putative proton channel.$^{75,77}$ In the H$_2$-evolution reaction, the inner circuit operating anticlockwise must be predominant, because pH$_{opt}$$^+$ lies below the pK$_a$. NiSI$_{1914}$ form receives electrons and H$^+$ successively to produce NiC$_{1914}$, then Ni-R$_{1914}$ and finally releases H$_2$ to regenerate Ni-SI$_{1914}$ to complete the cycle.

In the isotopic exchange, only the right half semi-circuits are operating with both of Ni-SI forms, because the pH optimum for the exchange lies near the pK$_a$. Both of the Ni-R forms are the molecular species which have properties compatible with the postulated H$_2$-adduct of the enzyme, EH$_a$Hb,i in Fig. 1,$^6$ where H$_a$ is suggested to be H bound to S-Cys*, because the temperature factor of this residue is found to be unusually high compared to the other side chains in the protein by X-ray crystallography.$^{57}$ Since Cys* locates at the entrance to the putative proton-channel to the protein surface,$^{75,77}$ the bound H on S-Cys* must have higher exchange rate than medium D$^+$ than the bridging hydride between Ni and Fe, which is H$_b$. NiFeSe$_2$-hydrogenase has Se$^+$-Sec instead of S$^+$-Cys*. As selenol is more acidic than thiol, Se$^+$-Sec would reduce the anionic character of the bridging hydride to make it more readily exchangeable with medium D$^+$ compared to S$^+$-Cys*.$^{73}$ i.e., k$_h$ of [NiFeSe]-enzyme would be higher than that of [NiFe]-enzyme, to make higher $\eta_{HD}/\eta_{VD}$ ratios shown in Table 2. In fact, only 3 fold increase in k$_h$ is sufficient to account for the $\eta_{HD}/\eta_{VD}$ ratio at pH 7.0 observed for [NiFeSe]-enzyme (Table 2), assuming the other kinetic constants unchanged. The proximal [4Fe-4S] cluster (not shown in Fig. 6), whose supporting residues are highly conserved among [NiFe]-hydrogenases (Fig. 2) is thought to be essential for the catalytic function. The catalytic mechanisms of [NiFe]-hydrogenase hitherto proposed$^{70,74,77,81,86,89,93}$ do not seem to aim at conforming to the results of kinetic studies$^6$ schematically presented in Fig. 1.

Concluding remarks

The enzyme hydrogenase has been spotlighted since its discovery, because controlled reaction of the substrate of the enzyme, H$_2$, is possible only in the
presence of catalyst, and the enzyme hydrogenase is expected to be a powerful tool to produce and utilize H₂ for future energy devices. Early achievements by studies on hydrogenases were the establishment of their electron carrier specificity and demonstration of heterolytic cleavage of H₂ on the enzyme. Our understanding on hydrogenases was remarkably widened and deepened since the elucidation of their three-dimensional structures by X-ray crystallography, and characterization of various states of the active site by spectroscopic studies such as EPR, FTIR etc. In this article, we surveyed the present knowledge on hydrogenases, and proposed the enzymatic mechanism by formulating the catalytic cycle running at the active site of the enzyme. This will provide a clue to design more efficient, oxidant-tolerant and stable catalyst by protein engineering, or to synthesize artificial catalysts for practical uses. Comprehensive reviews(7,75) are available for genetics, regulation or maturation of hydrogenases, not dealt with in this article.

Acknowledgements

We wish to express our gratitude to those who guided and encouraged us, especially to late Prof. Nobuo Tamiya and Prof. Hiroo Inokuchi, M.J.A., and to collaborators. Thanks are due to Prof. Tamio Yamakawa, M.J.A., who encouraged us to write this review article, and to Prof. Shigekazu Nagata, M.J.A., who communicated this paper at the meeting of the Japan Academy.

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(Received Sep. 20, 2012; accepted Nov. 1, 2012)

Profile

Tatsuhiko Yagi was born in 1933. He graduated from the Department of Chemistry, Faculty of Science, University of Tokyo in 1955. Discovery of carbon monoxide dehydrogenase was his first achievement as a graduate student of the same university. He moved to the Institute for Hard Tissue Research, Tokyo Medical and Dental University, as an assistant of Prof. Nobuo Tamiya in 1958. As a Fulbrighter he worked in the laboratory of Prof. Andrew A. Benson at the Pennsylvania State University, where he contributed to correct the structure of sulfolipid, which had been erroneously described. On returning to Japan in 1962, he started his life-work on hydrogenase with Prof. Tamiya. After obtaining the PhD degree on science, he moved to Shizuoka University in 1966, and was promoted to professor in 1972. His accomplishments there include, in addition to the studies on hydrogenase, elucidation of structure and function of bacterial electron carrier proteins such as cytochrome c₅₅, cytochrome c₋₅₅₃, ferredoxins and rubredoxin, discovery of multiheme high-molecular-weight cytochrome c, practice of an enzymic electric cell for the activity assay, discovery of 1-methoxyPMS as a versatile photostable electron mediator, etc. He helped Prof. Tamiya by reinforcing his hypothesis on evolution. He published many biochemical textbooks with him, and edited the Enzyme Handbook and an encyclopedia of compounds to search for enzymes. He is a councilor of the Japanese Biochemical Society, an emeritus member of the American Chemical Society and a member of the Chemical Society of Japan.
Profile

Yoshiki Higuchi was born in 1956 and started his research career in 1979 under the guidance of Prof. Masao Kakudo at Institute for Protein Research, Osaka University, and obtained his PhD degree for his X-ray crystallographic study on cytochrome c₅₅ in 1984. He was appointed as an Assistant Professor of Himeji Institute of Technology in 1985, moved to Kyoto University in 1995 as an Associate Professor of Graduate School of Science. He moved to Himeji Institute of Technology, as a Professor at the Graduate School of Science in 2002, and is currently a Professor at the Graduate School of Life Science, University of Hyogo. He has been involved in the studies on the structure and function of a [NiFe] hydrogenase from sulfate-reducing bacterium. He developed the purification and crystallization procedures of the hydrogenase and obtained the first single crystals in 1987, and succeeded in high resolution X-ray structural analysis of various forms with diverse activities of the same [NiFe] hydrogenase, a thorough investigation on a single object. At the same time, he successfully solved structure-function relationship on bacterial multi-copper oxidase, and other enzymes. He also solved the structure of the bacterial translational release factor, RF-3, complexed with a stringent factor, ppGpp. He received the Award of the Crystallographic Society of Japan in 1999. At present, he is involved in structural chemistry on hydrogenases including those from other sources, as well as on protein-protein interactions in the cells of higher organisms. He is a member of the Japanese Biochemical Society, the Chemical Society of Japan, the Protein Science Society of Japan, the Crystallographic Society of Japan, etc.