Role of Fe-hydrogenase in biological hydrogen production

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Fe-hydrogenase is a distinct class of hydrogen-producing metalloenzyme, present in a wide variety of prokaryotes and eukaryotes. It functions either in the utilization of hydrogen as a growth substrate (H_2 uptake) or in the disposing of excess electrons by combining them with protons to form hydrogen (H_2 evolution). The reversible conversion of molecular hydrogen (H_2) to protons (H^+) and electrons is a central reaction in the biological energy cycle of all hydrogen-producing organisms. The active centre of the Fe-hydrogenases contains a unique bimetallic (Fe–Fe) centre with non-protein ligands (CO, CN). X-ray crystallography and spectroscopic studies reveal that Fe-hydrogenase is a simpler system than the Ni–Fe counterpart. The present work summarizes the role of Fe-hydrogenase in the microbial production of hydrogen. Classification, distribution and localization of Fe-hydrogenase enzymes and their structural diversity have also been highlighted.

Keywords: Fe-hydrogenase, Fe–S protein, fermentation, hydrogen, H-cluster.

THE potential use of microorganisms for biological production of hydrogen as a future energy resource makes hydrogen metabolism an emerging field of research. Hydrogenase (H_2ase) is the name given to the family of enzymes that catalyse the reversible oxidation of hydrogen into its elementary particle constituents, two protons (H^+) and two electrons:

$$2H^+ + 2e^- \leftrightarrow H_2.$$

In the light of this reaction, it is reasonable to postulate the bacterial production of hydrogen as a device for disposal of electrons released in metabolic oxidations through the activity of hydrogenases. These are a heterogeneous group of enzymes with different sizes, subunit compositions, metal contents and cellular localizations. On the basis of metal content of catalytic subunit, H_2ase can be grouped into two non-homologous classes – those containing only Fe at the active site, called Fe-H_2ase and those with Ni, Fe and sometimes Se, [Ni–Fe] H_2ase and [Ni–Fe–Se] H_2ase[1,2]. Initially Fe-H_2ase was presumed to be present in a limited number of bacteria and anaerobic living protozoa[3]. Subsequently, it was revealed that its distribution in eukaryotes is also quite significant. Genes bearing signatures of Fe-H_2ase are found not only in prokaryotes and lower eukaryotes, but also in the genome of higher eukaryotes like mammals, although the physiological activity of these proteins is yet to be found out.

The presence of the enzyme Fe-H_2ase in bacteria has been known for over 70 years[4]. The requirement of Fe for its activity was discovered[5] in 1950s. The function of the cytoplasmic enzyme is to remove excess reducing equivalents during microbial fermentation and that of the periplasmic enzymes in hydrogen oxidation[6]. The highly reactive nature of Fe-hydrogenase enzymes is evidenced by their extremely high turnover numbers, 6000 s^{-1} for *Clostridium pasteurianum* and 9000 s^{-1} for *Desulfovibrio* sp. These are almost 1000 times higher than the turnover number of nitrogenases[7]. The present article updates the role of Fe-hydrogenase in the biological production of molecular hydrogen. Attempts have also been made to highlight some of the salient aspects of classification, structural diversity and biochemical assay of Fe-H_2ase and its limitations.

Hydrogen metabolism

Hydrogen metabolism is probably one of the most fundamental processes of living systems. Transfer of electrons to hydrogenase with subsequent production of hydrogen is intimately related with the primary energy metabolism in different microorganisms. Formation of hydrogen occurs as a major physiological process in some hydrogenase containing organisms, which display anaerobic energy metabolism[8,9]. Salient metabolism patterns of different groups of hydrogen-producing organisms have been depicted in Table 1. In aerobic metabolism, electrons from substrate oxidation are transferred to oxygen as the ultimate oxidant. But in case of anaerobic metabolism, electrons released from anoxygenic catabolism are used by different terminal oxidants such as nitrate, sulphate and organic compounds derived from carbohydrates[10]. Several physio-

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Table 1. Energy metabolism patterns in H_2-producing organisms

Representative organism	Salient metabolism pattern	Energy source (anaerobic)
Strict anaerobes	Disposal of electrons from energy-yielding oxidations Extensive accumulation of reduced organic products Cell yields relatively low	Fermentation
Facultative anaerobes	Promotion of energy-yielding oxidations through removal of formate Close coupling of H_2 evolution with energy-yielding reaction	Fermentation
Photosynthetic organisms		
Purple non-sulphur bacteria	Cell yields remarkably high	Light
Purple sulphur bacteria	Reduced organic byproducts not produced in significant amounts	
Anerobically adapted algae	Reduced pyridine nucleotide is the electron donor.	

logical groups of bacteria can evolve hydrogen under anaerobic conditions. These include fermentative bacteria, sulphate-reducing bacteria and phototrophic bacteria. Under anaerobiosis, fermentative metabolism predominates over others. Most purple and green bacteria can produce hydrogen at high rates either in the dark or in the light. Photoproduction of H_2 by the phototrophic bacteria is a nitrogenase-dependent reaction. H_2 is only produced under anoxic condition when nitrogen source is limited[11–13]. Nitrogenase is responsible for nitrogen-fixation[14] and is distributed mainly among prokaryotes, including cyanobacteria[15]. Nitrogenase-catalysed hydrogen production occurs as a side reaction at a rate of one-third to one-fourth that of nitrogen fixation, even in a 100% nitrogen gas atmosphere. Molecular nitrogen is reduced to ammonia via an irreversible reaction with consumption of electrons released by ferredoxin and ATP:

$$N_2 + 8H^+ + 2e^- \rightarrow 2NH_3 + H_2,$$

$$12ATP \rightleftharpoons 12ADP + 12Pi.$$

However, nitrogenase catalyses proton reduction in the absence of nitrogen gas with concomitant utilization of ATP.

$$2H^+ + 2e^- \rightarrow H_2,$$

$$4ATP \rightleftharpoons 4ADP + 4Pi.$$

Under normal growth conditions, cyanobacteria (blue-green algae) undergo photosynthesis (as in plants) in the light and can use water as the primary electron donor[16]. Both cyanobacteria and green plants have two photosystems. Production of H_2 in these bacteria is solely dependent on nitrogenase. But heterocystous cyanobacteria (e.g. *Anabena* sp. and *Nostoc muscorum*) can produce H_2 in the light without the interference of CO_2 fixation, O_2 evolution and sugar synthesis[17]. In all nitrogen-fixing bacteria, nitrogenase is accompanied with hydrogenase. Benemann and Weare[18] have demonstrated that a nitrogen-fixing cyanobacterium, *Anabaena cylindrica*, can produce hydrogen and oxygen gas simultaneously in an argon atmosphere for several hours. Such group of bacteria contains two major proteins, hydrogenase and ferredoxin in addition to nitrogenase, to shuttle electrons from the photosynthetic membranes to the hydrogenase (Figure 1). However, the efficiency of the process is lowered by high ATP requirement of nitrogenase in these bacteria[19,20]. Green algae and cyanobacteria both possess reversible hydrogenase with low energy requirements and the manner in which they produce hydrogen is called direct biophotolysis[21,22]. The reductant produced from the photosystems is transferred through reduced ferredoxin to hydrogenase.

Classifications of H_2ases

The first isolated and characterized H_2ase was found to be monomeric Fe–S protein[23]. Initially the classification was only based on the identity of specific electron donors and acceptors, quaternary structures, size and so on. According to the protein sequence homology of thirty sequenced microbial H_2ases, the enzymes can be classified into five groups:

(i) The first group contains H_2 uptake membrane bound [Ni–Fe]-H_2ases from aerobic, anaerobic and facultative anaerobic bacteria[24].

(ii) The second group comprises membrane bound H_2 uptake [Ni–Fe–Se]-H_2ase from sulphate-reducing bacteria[25].

(iii) The third consists of periplasmic Fe-H_2ase from strict anaerobic bacteria mainly responsible for hydrogen evolution[23].

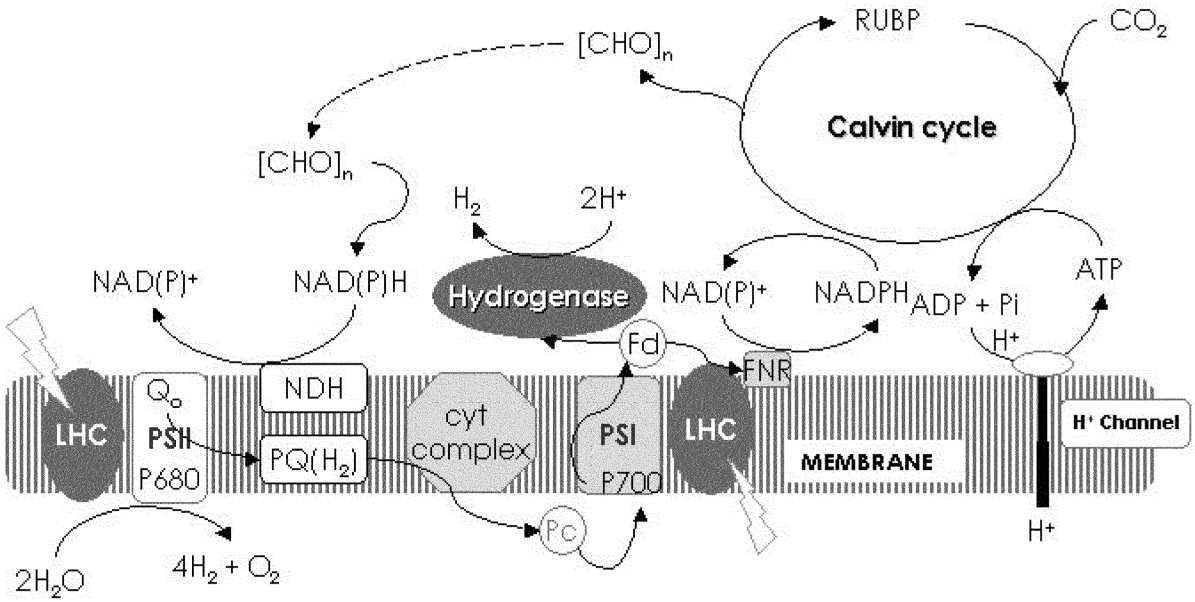

Figure 1. Nitrogenase-mediated hydrogen production in cyanobacteria[18].

Table 2. Classification of H_2ases

Classification	Occurrence/source	Structure	Features	
			Localization	Function
Ni–Fe-H_2ase	Anaerobic, photosynthetic bacteria, cyanobacteria	Heterodimeric, multimeric	Membrane-bound, cytoplasmic, periplasmic	Uptake of hydrogen
Ni–Fe–Se-H_2ase	Sulphate-reducing bacteria, methanogenes	Oligomeric	Membrane-bound, cytoplasmic	Oxidation of hydrogen
Fe-H_2ase	Photosynthetic bacteria, anaerobic fermentative bacteria, cyanobacteria, green algae, protozoan	Monomeric, heteromeric	Cytoplasmic, mambrane-bound, periplasmic, chloroplast, hydrogenosomes	Production of hydrogen
Metal-free H_2ase	Methanogens	Monomeric	Cytoplasmic	Formation of hydrogen

(iv) The fourth contains methyl viologen factor [F-420] or NAD-reducing and soluble H_2ases from *Methanobacteria* and *Alcaligenes* [26].

(v) The fifth is the labile H_2ase isoenzyme of *Escherichia coli*[27].

The results of the sequence comparisons reveal that these H_2ases share some common sequences. Those of classes (i), (ii), (iv) and (v) are homologous and share the same evolutionary origin. Despite increasingly conspicuous diversity in many respects, H_2ase can be classified broadly into three distinct classes: Ni–Fe H_2ase, Fe-H_2ase and metal-free H_2ase.

The vast majority of known H_2ases belong to the first class (Table 2) and over hundreds of these enzymes have been characterized genetically and/or biochemically[26–28]. Metal content as well as sequence similarity is a reliable classification criterion. Each of these classes is characterized by a distinctive functional core and is conserved within each group. Such phylogenetically independent classes of H_2ases get support from X-ray crystallography at least in the cases of [Ni–Fe]-H_2ases[28] and Fe-H_2ases[29]. Metal-free H_2ase[26] is found to be present in some methanogens[26], but the paucity of sequence data for metal-free H_2ase fails to give detailed information on the enzyme.

H_2ase assay

H_2ase activity may be assayed either by the reduction of electron carriers, e.g. methylene blue, benzyl viologen and methyl viologen, spectrophotometrically or by the Clark-type electrode method. But a simplified assay of hydrogenase involves an enzyme-catalysed reaction, which includes hydrogen production from common sugar, glucose using two enzymes, glucose dehydrogenase (GDH) and hydrogenase. GDH is an enzyme that oxidizes glucose to gluconic acid. GDH requires $NADP^+$ for its activity. Re-

duced NADPH is further oxidized to $NADP^+$ by hydrogenase isolated from any microorganism with the evolution of hydrogen. This hydrogen evolution can be detected with the red-ox dye benzyl viologen (BV), that turns purple when reduced. The absorbance is measured spectrophotometrically at a wavelength of 600 nm. The molar extinction coefficient of BV is 7400 at 600 nm. The stoichiometry of the reaction is one mole of glucose reduces one mole of BV; so by measuring absorbance at 600 nm, the amount of hydrogen produced can be estimated[30]. Hydrogenase activity of the bacterially expressed and purified protein can also be assayed spectrophotometrically using methyl viologen (MV) or NADH substrate (Figure 2). One unit of hydrogenase activity is the amount of enzyme that catalyses the reduction of 1 μM of MV or NADH per min, or the production of 1 μM of H_2 per min.

Evidences for involvement of Fe-H_2ase in hydrogen production

The genes for Fe-H_2ase were the first to be cloned and sequenced for *Desulfovibrio vulgaris* and the enzyme was purified to homogeneity[24,31]. Experimental results showed that in *D. vulgaris*, following a transfer of a broad-range host plasmid that constitutively expresses *hydAB* (Fe-H_2ase encoded gene) anti-sense mRNA, causes two to threefold reduced content of Fe-H_2ase[31]. The strain with reduced content of Fe-H_2ase showed less H_2 production compared to the wild-type *D. vulgaris*. Subcellular fractionation, immunochemistry, Western blotting and comparison of the deduced amino acid sequence of H_2ase available to date confirmed the presence of Fe-H_2ase in

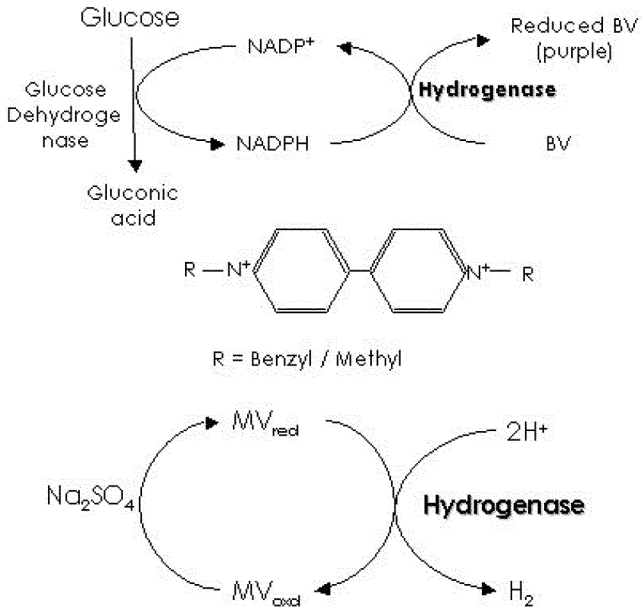

Figure 2. Reaction outline for measurement of hydrogen production in colorimetric assay[30].

the hydrogenosome of anaerobic chytrid[32], *Neocallimastix* sp. L2. This is the enzyme responsible for at least 90% of H_2 production of hydrogenosome, and its activity can be blocked by carbon monoxide. Gaffron and Rubin[33] first reported that a green alga, *Scenedesmus*, produced molecular hydrogen after being kept under anaerobic and dark conditions. Under anaerobic atmosphere, hydrogen metabolism is the only pathway for algae to create high amount of ATP, which is required for survival under stress conditions. Sulphur-deprivation in *Chlamydomonas reinhardtii* brings about prompt degradation of Rubisco (principal enzyme involved in photosynthesis) and substantial accumulation of starch. Thus the organism switches over the photosynthetic pathway to hydrogen metabolism[16,22]. Starch accumulation and subsequent breakdown of Rubisco provide the endogenous substrate that supports H_2-production, both directly by feeding electrons into the plastoquinone pool in chloroplasts, and indirectly by sustaining mitochondrial respiration for the maintenance of anaerobiosis in the cell[17]. Green algae respond to anaerobic stress by switching the oxidative pathway to the fermentative metabolism. Fermentation is mostly associated with hydrogen evolution. It has also been reported that the key enzyme, Fe-H_2ase is synthesized only after an anaerobic adaptation in *C. reinhardtii*. Results of the suppression subtractive hybridization (SSH) approach[34] showed that the Fe-H_2ase gene is differentially regulated under anaerobiosis only. All these compelling evidences from structures, sequences and experimental results suggest that Fe-H_2ases are the distinct class of H_2ases that is the only responsible enzyme in hydrogenesis.

Structural diversity

X-ray crystallography, spectroscopy and other biochemical studies have contributed significantly to the structural studies of Fe-H_2ase. The molecular architecture of the catalytic site of the enzyme has been resolved only with the X-ray crystal structure. Despite the wide occurrence of the Fe-H_2ase in prokaryotes and eukaryotes, the structure of only two Fe-H_2ases has been solved so far – from cytoplasm of *Clostridium pasteurianum* (CpH_2ase)[29] and periplasmic space of *Desulfovibrio desulfuricans* (DdH_2ase)[35]. The three-dimensional structure of CpH_2ase has been determined to 1.8 Å resolution by X-ray crystallography using multi-wavelength anomalous dispersion (MAD) phasing[29]. These data show that the larger domain of the enzyme possesses the catalytic activity termed as H-cluster and the apoprotein part of the enzyme constitutes some multiple copies of [2Fe-2S/4Fe-4S] clusters, known as the F-cluster. The molecular architecture of the H-cluster was confirmed by FTIR, EPR and IR studies (Figure 3 *a–c*). Such spectroscopic data indicate that the H-cluster of the Fe-H_2ase consists of a bimetallic centre with two Fe atoms (Fe_1–Fe_2). Each Fe of the binuclear centre

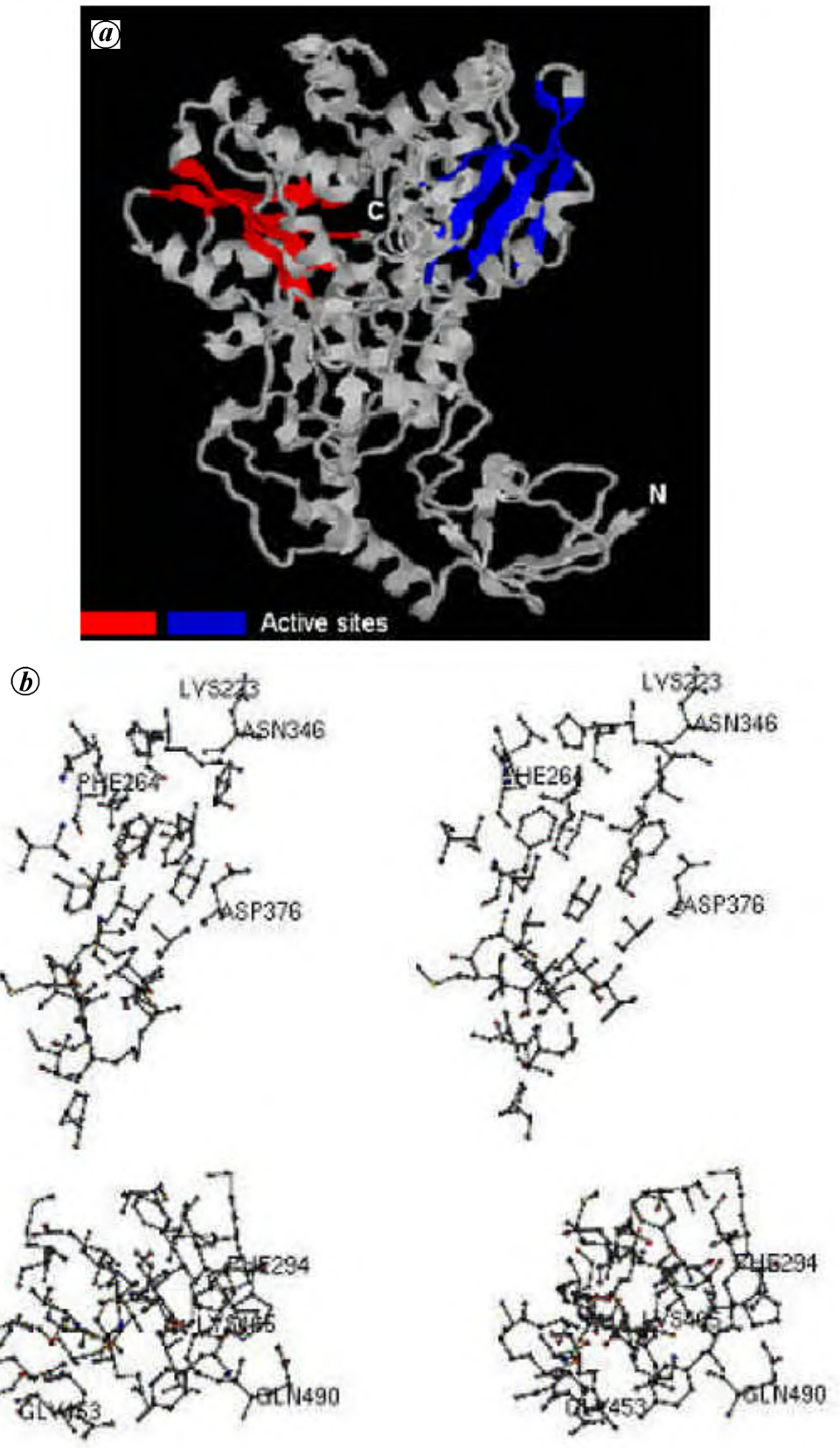

Figure 3 ***a***, ***b***. ***a***, Crystal structure of Fe-H_2ase from *Clostridium pasteurianum*. The active site domain of CpH_2ase consists of two four-stranded twisted beta sheets, each of which is flanked by a number of alpha helices and forms two equivalent lobes. The left lobe of the active site domain (red-coloured region) possesses four-stranded parallel beta strands and the right lobe (blue-coloured region) of the same contains four-stranded mixed three sheets. The active site cluster is located in the specified cleft[29]. ***b***, Stereo view of active site domain generated by MOLSCRIPT v.2.1.2.

Figure 3 *c*, *d*. *c*, Secondary structure prediction of CpH_2ase. *d*, Proposed structure of active site, H-cluster of CpH_2ase. H-cluster consists of binuclear Fe atoms bridged to a [4Fe–4S] subcluster through a sulphur residue of cysteine. Fe atoms are coordinated to CO/CN ligands and *X* may be a H_2O/CO ligand.

is coordinated to diatomic ligands like CO and CN. The two Fe atoms (Fe_1–Fe_2) in CpH_2ase are bridged by CO and in DdH_2ase by 1,3-propanedithiol-like bonds[36,37].

The coordination of strong-field diatomic ligands like CO and CN to the metallic centre makes Fe low spin and stabilizes at low oxidation state. Such low oxidation state at the Fe site facilitates the binding of hydrogen[38–40] as ligands and acts as a site to donate and accept protons for heterolytic mechanism of hydrogen production. The F-cluster and the non-coordinated Cys residue at the active site accommodate the electron and proton transfer pathways and form a putative channel for the access of hydrogen to the active site[41], and are responsible for electron transfer path from buried active site to the exterior of the protein. CpH_2ase consists of a single polypeptide chain and the structure resembles a mushroom. The large C-terminal domain contains the catalytic centre and makes up the cap of the mushroom and is bridged to the proximal F-cluster via the sulphur atom of the cysteine residue. Fe-H_2ase from *D. desulfuricans* possesses two polypeptide chains. The larger subunit contains the catalytic site, H-cluster and is covalently attached to the loops of beta-sheets at the active site domain. The most conserved part of H-cluster domain of Fe-H_2ases includes four cysteine ligands at the metal site, with a few residues like methionine or histidine lining the active site. This region exhibits high degree of conservation in sequence alignment of all Fe-only hydrogenases reported so far (Figure 3 *d*). Mössbauer and FTIR spectroscopic data[42,43] suggest that Fe_1 of the bimetallic centre has six ligands in distorted octahedral conformation, whereas Fe_2 has five ligands. CpH_2ase contains additional water as ligand, but DdH_2ase has an empty site. This apparent open site most likely binds hydrogen and is confirmed by the loss of activity of the enzyme by irreversible binding of both CO and CN at this site. Interpretation of EPR data and stretching frequencies of FTIR data explain well this phenomenon of loss of activity of H_2ase in the presence of CO/CN and its susceptibility towards oxygen with the plausible conformational changes that might occur at the H-cluster site upon oxidation and on reductive states[44].

Accessory domain(s)

In addition to the H-cluster domain, an N-terminal domain homologous to the bacterial ferredoxin [4Fe–4S] and/or bacterial thioredoxin [2Fe–2S] is also present in many Fe-H_2ases. *Trichomonas vaginalis* and *Megasphaera elsdenii* possess two monomeric subunits as accessory domain. *Clostridial*-type H_2ase is of 64 kDa, with three domains in addition to the H-cluster domain. *Desulfovibrio fructosovorans* H_2ase resembles *Clostridial*-type. The catalytic subunit of *Thermotaga maritima* is large (73 kDa), but its C-terminal domain is homologous to Nuo E subunit of NADH-ubiquinone oxido-reductases. The largest catalytic subunit so far reported is the monomeric H_2ase of anaerobic eukaryote, *Nycotherus ovalis*[1].

On the contrary, N-terminal part of eukaryotic Fe-H_2ase shows heterogeneity, which is lesser among bacterial Fe-H_2ases. Regarding the structural configuration, in most bacteria four motifs are implicated in the coordination of one [2Fe–2S] and three [4Fe–4S] accessory clusters[29]. In contrast among eukaryotes, *T. vaginalis* and *N. ovalis* contain a coordination of four putative accessory [Fe–S] clusters[45], surprisingly, green algae like *Chlorella, Chlamydomonas* and *Scenedesmus*[22,46] lack accessory domains though H_2 evolution and biochemical results are similar to *C. pasteurianum*.

Red-ox partners of Fe-H_2ase

Fe-H_2ases are efficient users of a wide range electron donors or acceptors. However, they differ greatly in size and number of accessory domains, which determine the selectivity of enzymes towards the red-ox partner. Structural information on the recognition of its red-ox partners is essential to understand the structure–function relationships of the enzyme. Considerable divergence is reached at the level of natural red-ox partners of H_2ases. Flavodoxins, ferredoxin, rubredoxins, monoheme cytochromes, and multi-heme cytochromes NADH, NADPH have all been implicated as putative partners of H_2ase (Table 3). This multitude defines electron transfer to and from H_2ase as a research area in itself. The red-ox partner can change in response to nutrient variation (the flavodoxin/ferredoxin switch depending on available Fe levels) or to a changing function (high-potential/low-potential partner depending on whether H_2 should be consumed or produced)[22,45]. Different H_2ases in the same cell can use different red-ox partners, and one particular H_2ase can be red-ox connected to different streams of metabolism. All this switching and branching of electron transfer requires research for enzymological, physiological and technological implications. Their occurrence in diverse organisms is endowed with a wide range of metabolic capabilities. Fe-H_2ase is versatile with respect to electron donors and acceptors. The metabolism of *M. elsdenii* is closely related to the *C. pasteurianum.* These bacteria use 4Fe–4S ferrodoxin under rich iron condition but flavodoxin during iron starvation[47]. In green algae, e.g. *Scendusmus obliquus*, *C. reinhardtii* and *Chlorella fusca*, the electrons for H_2 evolution are provided by the fermentative metabolism via plastoquinone pool into the photosystem-I (PS I) which in turn reduces plant-type [2Fe–2S] ferrodoxin[46]. In *D. fructosovorans* and *T. maritima*, NADP plays a physiological role in the functioning of the enzyme. The only correlation between accessory domains and red-ox partner specificity appears to be with the presence of Nuo-E and Nuo-F-like domains (subunits of NADP ubiquinone: oxidoreductase) in Fe-H_2ases interacting with NADP[48].

Table 3. Characteristics of Fe-hydrogenase

Organism	Size (no. of amino acid residues)	Red-ox partners	Approximate mol. wt. of Fe hydrogenase (kDa)
Clostridium pasteurianum	574	Fd, Fv, plant Fd	60–64
Megasphaera elsdenii	484	Fd, Fv	57–59
Desulfovibrio vulgaris Hildenborough	421–606	Fd, Fv, plant Fd	50
D. desulfuriacans	422	Fd, Fv, plant Fd	50
D. fructosovorans	421–585	Fd, Fv, plant Fd	NA
Escherichia coli	NA	Fd, Fv	64
Pyrococcus furiosus	NA	NAD(P)H, Fd	65
Thermotaga maritima	300–645	Fd, Hfd, Fv	NA
Chlamydomonus reinhardtii	497	Plant Fd, plastoquinone	53
Chlorella fusca	436	Plant Fd, plastoquinone	NA
Scendusmus obliquus	448	Plant Fd, plastoquinone	49
Nyctotherus ovalis	1206	Fd, Fv, Hfd, NE, NF	130
Neocallimastix sp. L2	NA	Fd, Fv, plant Fd	66.5
Trichomonas vaginalis	450–590	Fd	51.5–53
Giardia intestinalis	468	Fd	49

NA, Not available; Fd, Ferredoxin; Fv, Flavodoxin; NADP(P)H, Nicotinamide adenine dinucleotide phosphate (reduced form); Hfd, His ligand ferradoxin.

Distribution and localization of Fe-H_2ase

Extensive genomic sequencing efforts have currently revealed unexpected evolutionary connections among phylogenetically distant organisms. One of the most surprising examples is the presence of Fe-H_2ase in eukaryotic organisms like yeast, few anaerobic fungi, some ciliates, parabasalid flagellates, some micro aerophillic protozoa, parasite, diplomonad spiro nucleus, green algae, plants and even in mammals, including humans[3]. However, there are differences of opinion over the existence of H_2ases in various eukaryotes. The putative gene encoding H_2ase from each of these groups is cloned and characterized at the molecular level. The degree of the amino acid sequence homology between H_2ases and several homologous eukaryotic putative proteins is much higher than those previously reported. However, there is now evidence that sequences bearing common ancestry with Fe-H_2ases are located in aerobic eukaryotes, including humans. These genes are termed as NARF (nuclear prelamin A recognition factor) and display extensive similarity to Fe-H_2ase, specially with respect to the conservation of residues implicating in the coordination of the unique active H-cluster, as reported from two hybrid techniques. Human NARF protein interacts with prelamin A, the precursor form of a protein involved in the maintenance of the structural integrity of the nucleus[49,50]. In yeast, deletion of NARF-like gene is lethal in the haploid background[51]. Nuclear localization of NARF as well as the absence of reports on hydrogen production by higher eukaryotes suggests that these proteins are involved in energy metabolism. But clusters of Fe–S can facilitate electron transfer and contribute to the catalytic activity or help in maintaining the structural integrity of proteins. Proteins containing Fe–S are found to be involved in the sensing of oxidative stress, and also in red-ox dependent regulation of gene expression in prokaryotes. Processing and cleavage of nuclear lamins play a key role in programmed cell death (apoptosis) of higher eukaryotes[52]. Thus it can be speculated that such NARF-like protein may be involved in a variety of eukaryotic cellular processes, including cell cycle. Thus the proposition that Fe-H_2ases play a crucial role in eukaryogenesis, of both the new premises, the hydrogen hypothesis[53] and the syntrophic hypothesis[54] is found to be true. Furthermore, in eukaryotic fungi, e.g. *Neocallimastix frontalis*, *Trichomonads*[55,56] and in anaerobic ciliates, e.g. *Nyctothermus ovalis*, the enzyme Fe-H_2ase is present in the organelle called hydrogenosome, a peculiar organelle that supplies ATP and also produces molecular hydrogen by a similar mechanism like some eubacteria[32]. Hydrogenosome is generally derived from mitochondria, which in turn is originated from the free-swimming proteobacteria. But *Giardia intestinalis*, a eukaryote without hydrogenosome, produces hydrogen[32,57]. Under strictly anaerobic conditions, a mass spectrometric investigation of gas production indicates a low level of generation of hydrogen gas about tenfold lower than that in *T. vaginalis*, under similar conditions. In green algae, *C. fusca*, *S. obliqus*, *C. reinhardtii*[57], the enzymes are located in the chloroplast stroma and are linked via ferredoxin to the photosynthetic electron transport chain[34,46].

Improvement of hydrogenase activity – some approaches

Hydrogen metabolism in different microorganisms involves a coordinated action of two enzymes: nitrogenase and H_2-ase. However, the yield of hydrogen in hydrogenase-catalysed reaction is much higher than that of nitro-

genase-catalysed reaction[7]. Genetic studies on fermentative microorganisms have markedly increased, but relatively few genetic engineering studies have focused on altering the characteristics of these microorganisms, particularly with respect to enhancing the hydrogen-producing capabilities compared to photosynthetic bacteria and cyanobacteria. Different strategies can be followed for the amelioration of biological hydrogen production.

Development of oxygen-tolerant H_2ase

Among the H_2ases, Fe-H_2ase is an extremely oxygen-sensitive enzyme. In algal systems, H_2 photo-production ceases abruptly if oxygen is present. Oxygen inactivation is thought to occur by the direct binding of O_2 to one of the iron species (with an unoccupied coordination site) located at the catalytic centre[15]. Thus several precautions are required during purification of H_2ases. Sustained hydrogen generation is only possible if the enzyme H_2ase remains active in all physiological conditions, aerobic and anaerobic. Different strategies for surmounting the O_2-sensitivity problem include: (i) molecular engineering of the hydrogenase to remove O_2 sensitivity, and (ii) development of physiological means to separate O_2 and H_2 production. The approach to overcome oxygen sensitivity is only through classical genetics, either by site-directed mutagenesis or point mutation at *hyd*A (encoding Fe-H_2ase) gene. The focus of this approach is to identify the region, the particular amino acid residue, where the oxygen irreversibly binds and substitution of the residue may result in O_2 tolerance. Mutant prokaryotic organism, *Azotobacter vinelandii*[58] containing H_2ase with increased oxygen tolerance has been reported, which suggests that the enzyme is amenable to manipulations.

Repression of uptake hydrogenase

The presence of multiple forms of H_2ases in a single organism has been reported[59]. Hydrogen production via indirect photolysis using cyanobacteria can be improved by screening for wild-type strains possessing highly active hydrogen evolving enzymes (nitogenases and/or H_2ases) in combination with high heterocyst formation[60]. Genetic modification of strains to eliminate uptake H_2ases and increase levels of bidirectional hydrogenase activity may yield significant increases in H_2 production. For example, a mutant strain of Anabaena (AMC 414), in which the large subunit of the uptake hydrogenase (hupL) was inactivated by a deletion event[61], produced H_2 at a rate that was more than twice that of the parent wild-type strain, Anabaena PCC 7120. The uptake hydrogenase mostly contains Ni as the prosthetic group and is required for the assembly of the holo enzyme and also for its catalytic activity. If the cells are allowed to grow in Ni-deficient medium, synthesis of the uptake H_2ase will be blocked resulting in comparatively greater hydrogen evolution[15].

Metabolic shift

Disposal of excess reducing equivalents generated during fermentation is one of the major bottlenecks in facultative anaerobes which produce hydrogen[62]. These excess reducing equivalents could be disposed of via proton-reduction, facilitated by hydrogenase and electron carriers, leading to the formation of hydrogen in organisms such as *Enterobacter aerogenes*, *Enterobacter cloacae*, etc. In addition to volatile fatty acids, anaerobic fermentation also leads to formation of alcohols. These reduced end-products such as ethanol, butanol and lactate contain additional H atom that has not been liberated as gas[63]. Thus alcohol production gives correspondingly lower hydrogen yield. Therefore, to maximize the yield of hydrogen, the metabolism of bacteria must be directed away from alcohols (ethanol, butanol) and reduced acids (lactate) towards volatile fatty acids[64]. H_2ase, which consists of two subunits, interacts with NADH (reducing equivalent) on the cytoplasmic side and with protons on the periplasmic side. NADH is usually generated by catabolism of glucose to pyruvate via glycolysis. The conversion of pyruvate to ethanol, butanediol, lactic acid and butyric acid involves oxidation of NADH. The concentration of NADH would be increased if the formation of these alcoholic and acidic metabolites could be blocked[64]. This, in turn would augment the yield of hydrogen through the oxidation of NADH. The yields are reportedly[64] increased to 3.8 mol (mol glucose)$^{-1}$ by blocking the pathways of alcohol and organic acid formation by allyl alcohol and proton-suicide technique using NaBr and $NaBrO_3$. Similar enhancement of hydrogen yield using *E. aerogenes* HU-101 is reported by blocking the formation of alcoholic and acidic metabolites by allyl alcohol and also by proton suicidal technique[65,66].

Over-expression of Fe-H_2ase gene

Cloning and sequence analysis of Fe-H_2ase encoded gene (*hydA*) of many different fermentative bacteria and cyanobacteria is now under study. It is now possible to over-express the *hydA* gene from any hydrogen-producing organism into fast growing bacteria like *E. coli* under strong promoters[67,68]. Genetic manipulation with mutation at the transcription regulatory site can result in the constitutive expression of *hydA* gene, which was previously found to be active under derepressed condition only[69].

Conclusion

Hydrogenase research has been strengthened with X-ray crystallography and consequent simulations of other in-

vestigations. Complete genome sequences together with genetic and biochemical data indicate that Fe-H_2ases occur in bacteria and eukarya, but having distinct nature regarding accessory domains, size, red-ox partner specificity, charge distribution, etc. Discovery of H_2ase-like sequences in genomes of aerobic eukarya, including mammals implies the involvement of the enzyme in the evolution of eukaryotes, the hydrogenosomes and in the formation of eukaryotic cells, which can be an emerging track of H_2ase research. H_2ases of prokaryotic cells as well as of some eukaryotes are oxygen-sensitive. Thereby another future scope for the improvement of biological hydrogen production lies in the development of oxygen tolerant mutants.

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