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Review

[FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation ☆

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ABSTRACT

The [FeFe]- and [NiFe]-hydrogenases catalyze the formal interconversion between hydrogen and protons and electrons, possess characteristic non-protein ligands at their catalytic sites and thus share common mechanistic features. Despite the similarities between these two types of hydrogenases, they clearly have distinct evolutionary origins and likely emerged from different selective pressures. [FeFe]-hydrogenases are widely distributed in fermentative anaerobic microorganisms and likely evolved under selective pressure to couple hydrogen production to the recycling of electron carriers that accumulate during anaerobic metabolism. In contrast, many [NiFe]-hydrogenases catalyze hydrogen oxidation as part of energy metabolism and were likely key enzymes in early life and arguably represent the predecessors of modern respiratory metabolism. Although the reversible combination of protons and electrons to generate hydrogen gas is the simplest of chemical reactions, the [FeFe]- and [NiFe]-hydrogenases have distinct mechanisms and differ in the fundamental chemistry associated with proton transfer and control of electron flow that also help to define catalytic bias. A unifying feature of these enzymes is that hydrogen activation itself has been restricted to one solution involving diatomic ligands (carbon monoxide and cyanide) bound to an Fe ion. On the other hand, and quite remarkably, the biosynthetic mechanisms to produce these ligands are exclusive to each type of enzyme. Furthermore, these mechanisms represent two independent solutions to the formation of complex bioinorganic active sites for catalyzing the simplest of chemical reactions, reversible hydrogen oxidation. As such, the [FeFe]- and [NiFe]-hydrogenases are arguably the most profound case of convergent evolution. This article is part of a Special Issue entitled: Fe/S proteins: Analysis, structure, function, biogenesis and diseases.

1. Introduction

Hydrogenases utilize hydrogen (H_2) as a substrate or produce H_2 by the reduction of protons. The two main types of hydrogenases are classified by the nature of the metal clusters at their catalytic sites and are termed [FeFe]- and [NiFe]-hydrogenases [1–15]. Their active sites have structural characteristics in common including the presence of carbon monoxide (CO) and cyanide (CN^-) groups bound to the iron (Fe) ions. The [FeFe]-hydrogenase active site cluster is termed the H cluster, and exists as a regular [4Fe-4S] subcluster bridged to a 2Fe subcluster unit through a bridging cysteine thiolate. The 2Fe subcluster is also coordinated by the non-protein ligands carbon monoxide, cyanide and dithiomethylamine, thereby giving it a distinctly organometallic character. The Fe atom at the active site of the [NiFe]-hydrogenase has a similar architecture where one of the Fe ions of the 2Fe subcluster has CO and CN^- ligation but it is bridged to a Ni atom through bridging cysteine thiolates. The Ni atom is in turn terminally coordinated by two additional cysteine thiolates. In some hydrogenases one of the two terminal cysteines is replaced by selenocysteine and these so-called [NiFeSe]-hydrogenases are considered variations of [NiFe]-hydrogenases. Both [FeFe]- and [NiFe]-hydrogenases typically contain multiple iron-sulfur clusters that exist as either cysteine coordinated [4Fe-4S] or [2Fe-2S] clusters and in some cases there is mixed coordination with a His substituting for one of the Cys residues. As conduits between the catalytic metal sites and external electron donors and acceptors, the composition of these iron-sulfur clusters varies among different phylogenetic clades that in large part delineate hydrogenases on the basis of physiological function.

In the case of both [FeFe]- and [NiFe]-hydrogenases, the CO and CN^- ligands at their catalytic sites function as strong π-acceptor ligands that facilitate low-spin, II/I oxidation states of the Fe atoms. In all cases, the hydrogenases require efficient proton-transfer (PT), and proton-coupled electron transfer (PCET) to achieve fast H_2 activation rates

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turnovers ranging from ~10^3–10^4 s^{-1} [16–19]. This functional necessity is satisfied by a secondary coordination sphere that includes conserved polar residues with exchangeable sites for transferring protons to the redox-active bimetallic clusters [20–23]. In addition, these conserved residues can also participate in H-bonding interactions to stabilize cofactor orientations and tune electronic properties. Altogether these extended interactions are critical for creating a catalytic reaction coordinate that minimizes high-energy steps, or kinetic barriers that would otherwise constrain enzymatic function and efficiency.

For the most part, hydrogenases function in recycling reduced electron carriers that accumulate during anaerobic fermentation through proton reduction or in coupling H_2 oxidation to energy yielding processes. The [FeFe]-hydrogenases are typically associated with proton reduction and [NiFe]-hydrogenases with H_2 oxidation, although there are a number of exceptions [12,24]. Despite sharing the unique features of CO and CN— ligands to active site Fe ions, the [FeFe]- and [NiFe]-hydrogenases are not evolutionarily related and show no sequence similarity [25,26]. The only other example of an enzyme that exists with diatomic ligands to Fe is the methylenetetrahydromethanopterin dehydrogenase also termed Fe-hydrogenase found in methanogenic archaea. The Fe-hydrogenase has a single CO ligand to Fe and catalyzes the transfer of a hydride derived from H_2 to methenyltetrahydromethanopterin to form methylenetetrahydromethanopterin [27,28]. In general, [FeFe]-hydrogenases are found in anaerobic bacteria and are especially prevalent among the fermentative organisms (e.g., Firmicutes). They are also found in a number of eukarya including algae and protists but surprisingly they have yet to be found in cyanobacteria or in the archaeal domain. In contrast, [NiFe]-hydrogenases are frequently found associated with cyanobacteria and archaea, in addition to their common occurrence in a large number of bacteria. A variety of organisms harbor multiple hydrogenases [25,26,29,30]. In some cases, this may provide functional redundancy but it is thought in general they likely have different roles. Some bacteria, again largely firmicutes and sulfate reducing bacteria, even harbor both [NiFe]- and [FeFe]-hydrogenases [25,26].

The [FeFe]-hydrogenases are closely related to a protein found only in eukaryotes termed Nar1 or Narf [5,31]. This protein has been implicated in having a role in cytoplasmic iron-sulfur cluster biosynthesis and/or repair, and appears to resemble a minimal [FeFe]-hydrogenase that lacks a 2Fe subcluster. Homology models of Narf suggest the presence of an open cavity adjacent to the [4Fe-4S] cubane that could accommodate the 2Fe subcluster [5]. We have proposed [32] that Narf proteins and [FeFe]-hydrogenases have a common ancestor that more likely resembles the simpler Narf-like protein and that the biosynthetic pathway for assembly and insertion of the 2Fe subcluster evolved, at least in part, in response to the characteristics of the vacant cavity present in the ancestral enzyme and fill it with a organometallic cluster capable of catalyzing new biochemistry. Whatever the merits of this proposal, in general [FeFe]-hydrogenases do not occur universally in deeply rooted lineages of ribosomal RNA-based phylogenetic reconstructions and they are not commonly associated with metabolisms considered by many to be ancestral (e.g., chemolithotrophy or methanogenesis). This indicates that it is unlikely that [FeFe]-hydrogenases were a property of the Last Universal Common Ancestor (LUCA) and are perhaps not old from an evolutionary perspective [32].

As discussed below and in contrast to the [FeFe]-hydrogenases (Table 1), the [NiFe]-hydrogenases are encoded in the genomes of many Bacteria and Archaea and are frequently detected in deeply rooted lineages of both domains. For this reason, one can make an argument that [NiFe]-hydrogenases played a key role in the metabolism of the LUCA [33]. The closest evolutionary relative of [NiFe]-hydrogenases are proteins involved in respiratory Complex I/NADH dehydrogenase [34,35]. Subunits comprising NADH dehydrogenase are close relatives of the so-called group 4 membrane bound [NiFe]-hydrogenases that couple the oxidation of ferredoxin (Fd) to proton reduction, which is in turn coupled to generating a membrane potential through ion pumping. This mechanism is analogous to the mechanism of Complex I in coupling NADH oxidation and ubiquinone reduction to proton pumping. This suggests that ion pumping membrane [NiFe]-hydrogenases are the ancestors of modern respiratory Complex I and chemiosmosis.

That both [FeFe]- and [NiFe]-hydrogenases have independently evolved essentially the same unique solution to catalyzing reversible H_2 oxidation chemistry (carbon monoxide and cyanide ligands to Fe ions) is somewhat of a surprise. However, evidence for independent evolutionary origins is also supported in the stark difference in the mechanisms by which these unique carbon monoxide and cyanide ligands are synthesized in their respective cluster maturation pathways [36–41]. The process by which the CO, CN^-, and other nonprotein ligands are synthesized, coordinated to iron, and are inserted into the active sites of [FeFe]- and [NiFe]-hydrogenases to yield the mature enzymes, clearly represents two different paradigms in metal cluster biosynthetic pathways. These two independent paths for the evolution of these unrelated enzymes perhaps represents one of the most profound cases of convergence evolution and one that is supported by differences in the taxonomic distribution of these enzymes, the pathways required to synthesize active site cofactors, and their physiological roles.

2. Diversity

Our recent screening of 2919 complete bacterial and archaeal genomes available as of July, 2014 (Boyd, E.S., *unpublished data*) indicate the presence of [FeFe]-hydrogenase homologs in 265 of bacterial genomes (9.1% of total) while 778 (26.7% of total) of those genomes encode homologs of [NiFe]-hydrogenases (Table 1). Like previous studies [25,26,30], we found that [FeFe]- and [NiFe]-hydrogenase homologs are discretely distributed at the domain, phylum, and order levels of taxonomic classifications. While [FeFe]-hydrogenases are encoded in the genomes of anaerobic bacteria and anaerobic or phototrophic eukaryotes, they are not encoded by archaea. In contrast, [NiFe]-hydrogenases are encoded by both aerobic and anaerobic bacteria and archaea but not eukarya (Fig. 1).

2.1. Phylogenetic and functional diversity of [FeFe]-hydrogenases

[FeFe]-hydrogenase are delineated from paralogous proteins present in eukaryotes, such as Nar1, by the presence of three conserved cysteine-containing motifs that coordinate the active site H-cluster. These consist of TSCCPxW (L1), MPCxxKxxE (L2) and ExMxCxxGCxxG (L3) in [FeFe]-hydrogenase, and although some sequence variation does occur in these motifs, the cysteine residues shown in bold are essential for coordination of the H-cluster [25]. In addition to variation in the cluster binding motifs, previous studies have noted substantial sequence variation in the motifs that contain the cysteine residues that bind the N- and C-terminal Fe-S cluster and accessory cofactor binding domains [29,30] (Fig. 2), suggesting potential interactions with a variety of redox partners. Due to the substantial N and C-terminal cluster variation, only the H-cluster is included in phylogenetic reconstructions of the catalytic subunit, which is termed HydA. Although the branching order of some H-cluster lineages mirror that of taxonomic reconstructions, most organisms with *hydA* encode multiple copies that often belong to divergent lineages, implying a number of gene duplications and horizontal gene transfers during the functional diversification of this protein class. In addition to variation at the level of primary sequence, disparities in the number of [FeFe]-hydrogenase subunits and their tertiary structure have been identified [29,30].

The best characterized [FeFe]-hydrogenases are monomeric, ferredoxin-dependent enzymes [26]. Examples include the hydrogenases from algae, such as *Chlamydomonas reinhardtii*, and clostridial species, such as *Clostridium pasteurianum*. *C. reinhardtii* [FeFe]-hydrogenase HydA1 (*Cr*HydA1) contains only the H-cluster and no additional iron-sulfur clusters [30]. Like bacteria, algae often encode multiple homologs of [FeFe]-hydrogenase. However, unlike bacteria, homologs in a given algal taxon are monophyletic relative to other algal or bacterial

Table 1
Taxonomic distribution of [FeFe]- and [NiFe]-hydrogenase homologs in 2912 sequenced archaeal and bacterial genomes.

Domain	Phylum	Total number of genomes	Number of genomes with homologs	
			[FeFe]-hydrogenase	[NiFe]-hydrogenase
Archaea	Crenarchaeota	50	0	22
	Euryarchaeota	110	0	70
	Korarchaeota	1	0	1
	Nanoarchaeota	1	0	0
	Thaumarchaeota	5	0	0
Bacteria	Acidobacteria	7	0	4
	Actinobacteria	301	8	72
	Aquificae	14	0	13
	Armatimonadetes	2	0	0
	Bacteroidetes	94	9	12
	Caldiserica	1	0	0
	Chlamydia	109	0	0
	Chlorobi	11	0	10
	Chloroflexi	24	11	20
	Chrysiogenetes	1	0	1
	Cyanobacteria	79	0	43
	Deferribacteres	4	0	4
	Deinococcus-Thermus	21	0	1
	Dictyoglomi	2	2	2
	Elusimicrobia	1	0	1
	Fibrobacteres	2	0	0
	Firmicutes	622	160	86
	Fusobacteria	11	2	0
	Gemmatimonadetes	1	0	1
	Ignavibacteriae	2	2	0
	Nitrospirae	4	1	2
	Planctomycetes	8	0	1
	Proteobacteria	1240	28	497
	Spirochaetes	65	20	0
	Synergistetes	5	2	3
	Tenericutes	89	0	0
	Thermodesulfobacteria	2	1	2
	Thermotogae	18	18	1
	Verrucomicrobia	5	1	2

HydA, implying independent gene duplications in each of the algal lineages [42]. Given the structural simplicity of the *C. reinhardtii* hydrogenase it has become the model for active site maturation studies ([32,43,44]). Phylogenetic analyses indicate that the H-cluster domain of algal [FeFe]-hydrogenase is not likely to be ancestral, rather it is likely that the ancestral enzyme had a single ferredoxin binding domain at the N-terminus which was lost when these genes were laterally transferred to algae [32]. The simple algal enzyme containing only the H-cluster is thought to function during the fermentation of carbohydrate reserves [45,46] although photosynthetic H_2 production was recently demonstrated under fully aerobic conditions in the alga *Chlorella vulgaris* [47].

C. pasteurianum contains [FeFe]-hydrogenases of the ferredoxin (Fd)-dependent monomeric (M3) structural category [9] (Fig. 2). *C. pasteurianum* ferments 3 mol glucose to 2 mol acetate, 2 mol butyrate, 4 mol CO_2 and 8 mol H_2. In addition, reduced ferredoxin (Fd_{red}) is generated by the bifurcating butyryl-CoA dehydrogenase, which helps to explain how 8 moles of H_2 are produced instead of the 6 mol that would be expected based on thermodynamic considerations [48]. The Fd-dependent [FeFe]-hydrogenases are widespread in anaerobic bacteria, in particular in the phylum Firmicutes [25,29]. More complex, multimeric [FeFe]-hydrogenases have been identified in *Thermotoga maritima*, *Thermoanaerobacter tengcongensis* and *Desulfovibrio fructosovorans* and these have been implicated as NAD(P)H-linked H_2 producing enzymes [49–51]. The trimeric hydrogenase of *T. maritima* was shown not to couple H_2 formation with the oxidation of reduced Fd or of NADH when each was used as the sole electron donor. However, the presence of both reduced Fd and NADH promoted efficient H_2 production [52]. The key to the formation of H_2 from Fd and NADH simultaneously is the coupling of their energetics; the exergonic reduction of protons by electrons derived from Fd allows the endergonic reduction of protons by electrons from NADH. This type of reaction mechanism is called

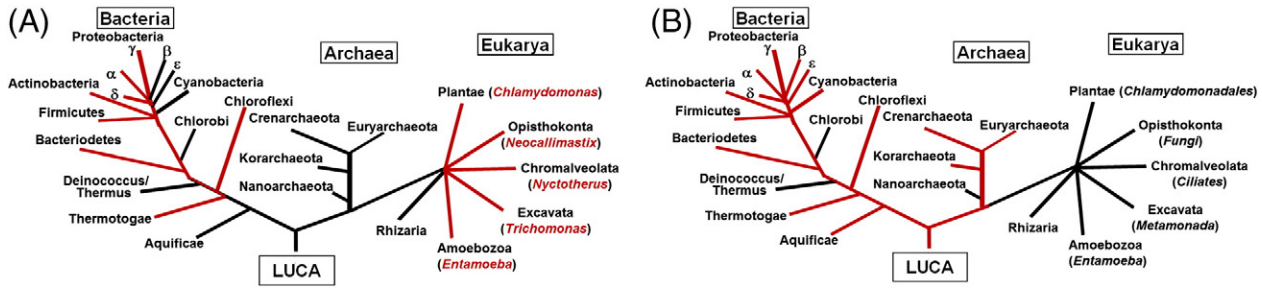

Fig. 1. Distribution of [FeFe]-hydrogenase (A) and [NiFe]-hydrogenase (B) mapped on a "universal" taxonomic tree of life. Lineages with at least one homolog of each enzyme are overlaid in red. Parsimony rules were used to map the ancestral character of extinct lineages (e.g., LUCA).

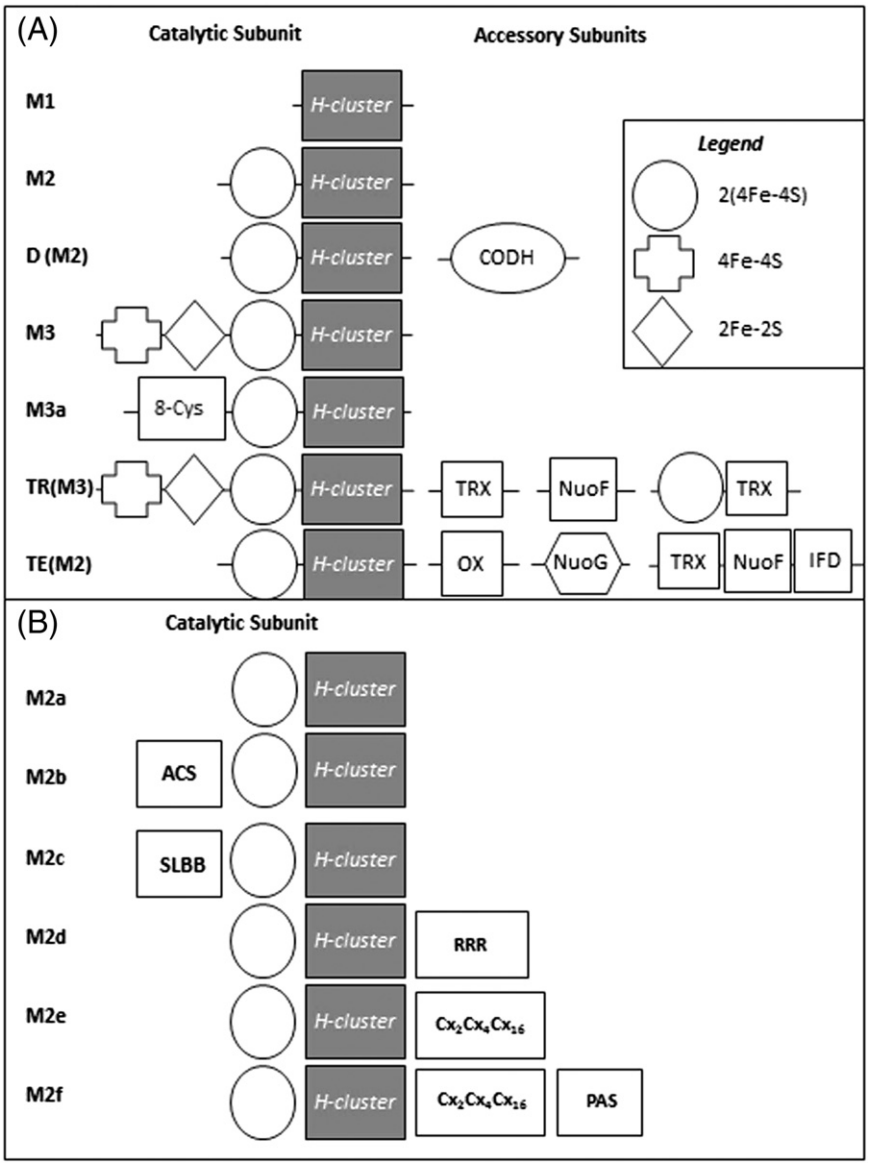

Fig. 2. Schematic representation of the structural variation of [FeFe]-hydrogenase homologs present in sequence databases as adapted from Meyer, 2007 and more recently Calusinska et al., 2010. The M1, M2, and M3, as reported by Meyer, 2007, correspond to monomeric enzymes with numbers indicating increasing size. "D" denotes a dimeric enzyme, "TR" denotes a trimeric enzyme, and "TE" denotes a tetrameric enzyme. Panel A illustrates the primary structural classes while panel B illustrates the remarkable structural variation within the M2 structural subclass. Abbreviations: CODH, carbon monoxide dehydrogenase; TRX: Thioredoxin domain; NuoF: homolog subunit F of NADH dehydrogenase; OX: oxidoreductase domain; NuoG: homolog of subunit G of NADH dehydrogenase; IDF: Indolepyruvate:ferredoxin domain; ACS: Acetyl CoA dehydrogenase; SLBB: soluble-ligand-binding b-grasp fold domain; RRR: Rubredoxin–rubrerhythrin–rubredoxin domain; PAS: PAS/PAC domain.

electron bifurcation and represents the third form of energy conservation along with substrate phosphorylation and electron transport phosphorylation [48,52].

Energy conservation by electron bifurcation was first proposed in 2008 [53] and is defined as an exergonic reaction driving an endergonic reaction without the involvement of an ion gradient. The clostridial cytoplasmic enzyme complex butyryl-CoA dehydrogenase catalyzes the reduction of crotonyl-CoA ($E^{0\prime} = -10$ mV) to butyryl-CoA with NADH ($E^{0\prime} = -320$ mV) as the electron donor and this is coupled to the endergonic reduction of Fd ($E_m = -410$ mV) [53,54]. Reduced Fd is then recycled to produce H_2 ($E^{0\prime} = -420$ mV). As a consequence of this pair of linked redox reactions, the oxidation of NADH can be coupled to H_2 production and this ultimately leads to the formation of additional ATP. The discovery of electron bifurcation can explain important reactions in anaerobic microbes, such as in clostridia and methanogens [55].

The heterotrimeric [FeFe]-hydrogenase from *T. maritima* (T_{opt} 80 °C) utilizes the exergonic oxidation of Fd [($E_m = -453$ mV (at 80 °C), [56,57]] to drive the unfavorable oxidation of NADH ($E^{\circ\prime} = -320$ mV) to produce H_2 ($E^{0\prime} = -420$ mV). The overall reaction is:

$$NADH + 2Fd_{red} + 3H^+ \rightarrow 2H_2 + NAD^+ + 2\ Fd_{ox}$$

Although technically this hydrogenase can be called confurcating as it merges electrons of different potentials, for simplicity reasons the term bifurcation should be used in all cases where 3 redox reactions are involved simultaneously, including this type of hydrogenase. The bifurcating hydrogenases provide new insights into H_2 production by anaerobes. For example, *Caldicellusiruptor* and other *Thermoanaerobacteriales* contain two types of hydrogenase, a soluble multimeric [FeFe]-hydrogenase similar to the *T. maritima* bifurcating hydrogenase and a group 4 membrane bound Fd-dependent [NiFe]-

hydrogenase. These enzymes were proposed to function together to evolve H_2 during sugar metabolism [50]. However, genetic analyses of *T. saccharolyticum* showed that the membrane bound [NiFe]-hydrogenase appeared not to be involved in H_2 production while deletion of the multimeric [FeFe]-hydrogenase resulted in decreased H_2 production [58]. This indicates that these organisms also utilize a bifurcating [FeFe]-hydrogenase for H_2 production and the non-bifurcating [NiFe]-hydrogenase could have a secondary metabolic role.

Bifurcating [FeFe]-hydrogenases also play a central role in energy conservation in acetogens [59]. *Acetobacterium woodii* uses a respiratory system for electron transfer between Fd and NAD as the main energy-conserving mechanism for autotrophic growth on H_2 and carbon dioxide [60,61]. However, the mechanism for how reduced Fd is generated from H_2 only became clear through the characterization of its bifurcating [FeFe]-hydrogenase, which functions to couple H_2 oxidation to the simultaneous production of reduced Fd and NADH [59].

Bifurcating [FeFe]-hydrogenases can in principle function *in vivo* in both the production and oxidation of H_2, as was recently shown for a reversible bifurcating hydrogenase in *Moorella thermoacetica* [62]. During growth on glucose, *M. thermoacetica* can intermittingly produce H_2 from Fd and NADH via the bifurcating hydrogenase. Likewise, this same hydrogenase can utilize produced H_2 and CO_2 to perform acetogenesis generating 3 mol of acetate per glucose molecule. Although *M. thermoacetica* does not grow well autotrophically on H_2 and CO_2, it uses the bifurcating hydrogenase to produce reduced Fd to drive acetogenesis. In addition to Fd- and NADH-dependent hydrogenases, a Fd- and NADPH-dependent enzyme was characterized from *Clostridium autoethanogenum* cultured on CO and it was proposed that this hydrogenase functions in the formation of H_2 [63]. Moreover, the genome of *C. autoethanogenum* encodes two additional [FeFe]-hydrogenases that from sequence and genomic context information are likely to have similar structure to known bifurcating hydrogenases and could function in providing reduced Fd and NADH during autotrophic growth on H_2 and CO_2 [64]. Bifurcating hydrogenases might also play an important role in the gut microbiota. *Ruminococcus* species, which are involved in the degradation of recalcitrant biomass, contain [FeFe]-hydrogenases very similar to the bifurcating hydrogenase of *T. maritima* [65]. Anaerobic gut-dwelling protists and fungi involved in biomass degradation also encode [FeFe]-hydrogenases to recycle their electron carriers that bear a similarity to the bifurcating [FeFe]-hydrogenases of anaerobic bacteria [66–68]

2.2. Phylogenetic and functional diversity of [NiFe]-hydrogenases

[NiFe]-hydrogenase are more widely distributed taxonomically than [FeFe]-hydrogenase (Table 1). Representative [NiFe]-hydrogenases have been isolated from a variety of microorganisms and biochemically characterized [69,70]. These enzymes are comprised of at least two subunits, with the large subunit (LSU) containing the NiFe-catalytic site and the small subunit (SSU) containing three highly conserved iron-sulfur clusters that serve to shuttle electrons between the external electron carrier and the NiFe site. Based on comparison of the sequence motifs that coordinate the active site, [NiFe]-hydrogenases can be separated into four distinct functional groups that in general correspond to their putative physiological role [26]. Intriguingly, phylogenetic reconstructions of representatives of these enzymes also reveal patterns of clustering that correspond to variations in NiFe cluster binding motifs and their inferred physiological role [26,29] (Fig. 3).

2.2.1. Group 1

Enzymes within this group are typically membrane-bound hydrogenases and are found in organisms that use H_2 as an energy source. A cytochrome *b* is often also present to anchor the hydrogenase complex to the membrane and allow electron transfer to the quinone pool. These enzymes couple the oxidation of H_2 to the reduction of electron acceptors such as oxygen, nitrate, sulfate, CO_2 or oxidized sulfur compounds [26]. *E. coli* contains 2 homologs of this group and one of these (Hya) is thought to oxidize H_2 and transfer electrons to the quinone pool to provide the cell with additional energy [71]. The sulfate reducing bacterium *Desulfovibrio gigas* encodes two [NiFe]-hydrogenases. One is a membrane bound Ech-type group 4 enzyme while the other is a periplasmic group 1 enzyme (HynAB). By mutational analysis it was shown that the periplasmic hydrogenase is essential for growth on H_2 with sulfate and functions to donate electrons from H_2 to the quinone pool via a periplasmic cytochrome c [72]. *Aquifex aolicus* is one the most thermophilic bacteria known and it gains energy by oxidizing H_2 using low concentrations of oxygen or oxidized sulfur compounds as electron acceptors [73]. Its genome encodes two trimeric uptake (group 1) [NiFe]-hydrogenases (MbhI and MbhII) and these have distinct physiological functions [74]. MbhI is involved in the oxidation of H_2 linked to oxygen reduction while H_2 oxidation by MbhII is coupled to elemental sulfur reduction [75]. A similar H_2-oxidizing and elemental sulfur reducing respiratory system was described in the archaeon

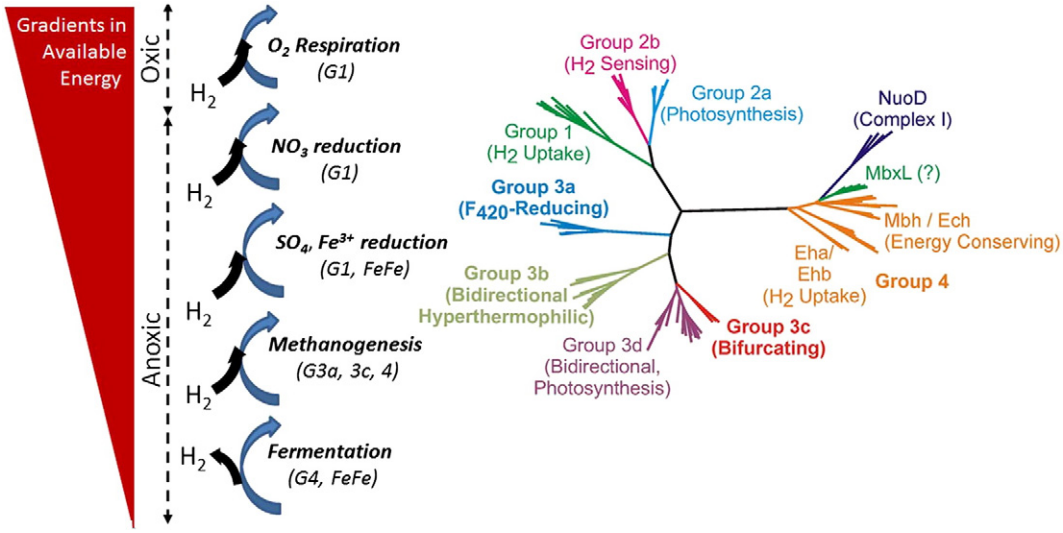

Fig. 3. Hypothetical depiction of the distribution of specific guilds of organisms and associated [FeFe]- and [NiFe]-hydrogenases (designated by "G") that function to couple the oxidation or reduction of substrates along a gradient in the availability of oxidants. (B) Phylogenetic reconstruction of representatives of [NiFe]-hydrogenase and related paralogs (NuoBD, MbxJL) with group designations indicated. Figure adapted from Boyd et al., 2014.

Acidianus ambivalens [76] but its genome sequence is not available and so the diversity of its hydrogenases is currently not resolved.

2.2.2. Group 2

2.2.2.1. Group 2a. This subgroup includes hydrogenases of cyanobacteria that fix dinitrogen (N_2) using nitrogenase. These organisms contain a dimeric uptake hydrogenase (HupSL) to recapture energy lost as H_2 during the reduction of N_2 [77]. Electrons from H_2 are then channeled into the quinone pool or function to reduce O_2 that deactivates the oxygen sensitive nitrogenase enzyme [78,79]. Mutation of the HupLS genes in the diazotroph *Anabaena siamensis* abolished H_2 uptake activity and led to an increase in light-dependent H_2 production, presumably due to the lack of recapture of H_2 produced by nitrogenase enzyme in the absence of the uptake hydrogenase [80].

2.2.2.2. Group 2b. Group 2b consists of H_2-sensing regulatory hydrogenases that function to signal the availability of H_2 to the transcriptional regulation of metabolic hydrogenases, which themselves are most often affiliated with Group 1 H_2 oxidizing enzymes [25]. A very well studied model is the H_2 oxidizing aerobe *Ralstonia eutropha*, which contains three different hydrogenases. In this case its H_2 sensing hydrogenase HoxBC regulates the expression of the membrane bound (HoxKGZ) and the cytoplasmic NAD-dependent (HoxFUYH) enzymes [81].

2.2.3. Group 3

2.2.3.1. Group 3a. The F_{420} reducing hydrogenases (Frh) are exclusively found in methanogenic archaea where they function in coupling the oxidation of H_2 to the reduction of cofactor F_{420}, thereby supplying reductant for two steps in the reduction of CO_2 to methane [25]. These enzymes consist of a large subunit (FrhA), a small subunit (FrhG), and a FAD containing-subunit (FrhB) for electron transfer to F_{420} [82,83].

2.2.3.2. Group 3b. Group 3b enzymes are tetrameric ($\alpha\beta\gamma\delta$) and are found primarily in thermophilic archaea. Their α and δ subunits represent the minimal [NiFe]-hydrogenase structure, with the two other subunits (β and γ) containing iron-sulfur clusters and a NAD(P)/FAD binding domain [26]. The enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus* has been well characterized and shown to function as an uptake hydrogenase to provide NADPH for biosynthesis, although mutational analysis has shown that it is not required for growth or for supplying NADPH to other metabolic processes under standard laboratory growth conditions [84–86].

2.2.3.3. Group 3c. Some archaea, primarily methanogens, encode a trimeric methyl viologen-reducing hydrogenase (MvhAGD). MvhAGD forms a complex with heterodisulfide dehydrogenase (HdrABC) that in turn provides the physiological electron acceptor heterodisulfide CoM-S-S-CoB [87]. With the discovery of electron bifurcation it became clear that the MvhADG/HdrABC complex catalyzes the reduction of heterodisulfide and Fd simultaneously with H_2 as reductant at high specific activity, while in the absence of Fd only low rates are observed [88]. The complex thus appears to couple the endergonic reduction of Fd ($E' \approx -500$ mV) to the exergonic reduction of CoM-S-S-CoB ($E'_0 = -140$ mV) with H_2 ($E'_0 = -414$ mV) as the electron donor, providing a putative mechanism by which methanogens conserve energy [55,89].

2.2.3.4. Group 3d. This subgroup consists of bidirectional heteromultimeric [NiFe]-hydrogenases (HoxHY) that are associated with an additional NADH oxidoreductase (diaphorase) module, which shows distinct homology to the NADH input module of Complex I [26]. The bidirectional hydrogenases are found in aerobic H_2-utilizing organisms such as *Ralstonia eutropha*, in which they most likely catalyze H_2 oxidation and supply reducing equivalents (NADH) to Complex I for energy generation or provide reductant for biosynthesis [90]. These bidirectional hydrogenases are also found in many cyanobacteria where they function to dispose of excess electrons derived from fermentation and photosynthesis [78]. In the cyanobacterium *Synechocystis* spp. PCC 6803 it was shown that the bidirectional hydrogenase could also directly accept electrons from Fd or flavodoxin, which explains the production of H_2 by over-reduction of the Fd pool in the light or fermentative metabolism under anoxic dark conditions [91].

2.2.4. Group 4

Group 4 [NiFe]-hydrogenases are all membrane-bound and are mostly Fd-dependent enzymes. These enzymes cluster distinctly from all the other hydrogenases indicating that they have a separate evolutionary history [25]. The members of this group fall into 3 major subgroups: 1) six subunit reversible ion-translocating enzymes termed Ech (energy converting hydrogenase), which are found in various orders of bacteria and a few archaea [34], 2) a thirteen (or even more) subunit membrane bound and ion-translocating enzyme (Mbh) with subunits homologous to Na^+/H^+ antiporters (Mrp) and these Mrp-Mbh enzymes are common in the thermophilic archaea [92], and 3) a variant of the Mrp-Mbh (Eha/ Ehb) present in hydrogenotrophic methanogens that contains additional subunits (17–20 subunits) and uses an ion gradient to reduce Fd with H_2 [93]. The group 4 hydrogenases also include a number of distinct H_2-evolving multienzyme complexes such as the formate hydrogen lyase of *E. coli* and *Thermococcus onnurineus* that oxidizes formate and evolves H_2 [94,95], and the CO-induced hydrogenases of *T. onnurineus* and *Carboxydothermus hydrogenoformans*, which are involved in generating energy from the oxidation of CO to CO_2 coupled with the production of H_2 [96,97]. The six-subunit energy converting hydrogenase (EchA-F) was originally characterized in *Methanosarcina barkeri* where it was found to be required for its growth on acetate [98]. This hydrogenase can reversibly generate H_2 from reduced Fd with the concomitant generation/utilization of a proton gradient; homologs of this enzyme are found among anaerobic bacteria and a few archaea. The Mrp-Mbh-type energy-converting H_2-producing enzymes were first described in the hyperthermophilic archaeon *P. furiosus* [92,99,100]. The striking difference between Mrp-Mbh and Ech is the presence of the Mrp-like H^+/Na^+ antiporter module in the Mrp-Mbh complex and the subsequent generation of a sodium gradient rather than a proton gradient. This Fd-dependent enzyme is linked to the glycolytic pathway found in heterotrophic archaea that is unique in that Fd is the only electron carrier (NADH is not produced). This allows for the efficient production of 4 mol of H_2 per mole of glucose [101] The energy converting hydrogenases of hydrogenotrophic methanogens (Eha and Ehb), are thought to provide reduced Fd for biosynthesis and to balance the first step of methanogenesis, the Fd-dependent reduction of CO_2 to form formylmethanofuran [93,102].

The group 4 hydrogenases also contain a subunit core that has close homologs in three non-hydrogenase enzyme complexes. These are termed cofactor F_{420} oxidoreductase (Fpo) found in methanogenic archaea, a membrane oxidoreductase (Mbx) involved in sulfur reduction by hyperthermophilic archaea such as *P. furiosus*, and NADH quinone oxidoreductase (Complex I, Nuo) found in aerobic bacteria and eukaryotes [35,52]. In particular, the close relationship between group 4 hydrogenases and components of Complex I of the ubiquitous aerobic respiratory chain highlights the intimate relationship between the evolutionary history of H_2 and oxygen respiration [35,52].

2.2.5. Group 5

Recently a group 5 [NiFe]-hydrogenase was proposed based on genome sequence, phylogenetic reconstructions, and biochemical characterization of an enzyme from *Streptomyces spp*. that is capable of oxidizing very low (<1 ppm) levels of H_2 [103–105]. It appears that group 5 hydrogenases are abundant among soil bacteria where they presumably provide the capability to take advantage of low H_2 concentrations [106,107]. Group 5 hydrogenases are similar in sequence structure to the group 1 enzymes in that they also consist of a small and large

subunit and associate with the membrane, but lack a potential cytochrome containing membrane anchor and as of yet have no identified redox partner. Thus, while appearing to be biochemically similar to group 1 H_2-oxidizing enzymes, the group 5 enzymes are distinct from the group 1 enzymes in that these are oxygen resistant enzymes and their [NiFe] binding motifs are different [103].

3. Hydrogenase mechanism

3.1. [NiFe(Se)]-hydrogenases

3.1.1. Catalytic site structure, coordination sphere and H_2 activation mechanism

X-ray structures of both [NiFe]- and [NiFeSe]-hydrogenases poised in various active and inactive states have been collected and these have provided detailed insights into cofactor arrangements and coordination environments [13,24,108–115]. The bimetallic cofactor of [NiFe(Se)]-hydrogenase is composed of Ni and Fe atoms bridged by a pair of Cys thiolates. Two CN- and one CO complete the coordination of the iron ion to create a subsite resembling the 2Fe subcluster of the [FeFe]-hydrogenase H cluster (Fig. 4). A second pair of Cys thiolates completes the coordination of the Ni atom, one of which is replaced by selenocysteine in [NiFe(Se)]-hydrogenases. Surrounding the Ni-Fe cluster are conserved Arg, His and Glu residues, the functional groups of which create a network of exchangeable sites near or within H-bonding distance to the Ni-Fe cluster illustrated in Fig. 4A. Mutagenesis of the nearby Arg, Glu and His residues leads to loss or attenuation of catalytic activity, consistent with their proposed function in proton transfer (PT) and/or H-bonding interactions with the [NiFe] cluster [116,117]. In addition, electron nuclear double resonance (ENDOR) spectroscopy and high-resolution crystallography have shown that the Se/S groups that coordinate Ni can function as a base to accept protons during PT and H_2 activation [6].

3.1.2. [NiFe(Se)-hydrogenase oxidation states under H_2 activation

Detailed summaries and overview of the various crystallographic, paramagnetic (e.g., EPR, ENDOR), and infrared (IR) spectroscopic properties of catalytic intermediates of [NiFe(Se)]-hydrogenases have recently been extensively reviewed [6,12,118,119]. There are several reasonably defined catalytic states observed in the different classes of [NiFe(Se)]-hydrogenases (Table 2). Notwithstanding the significant experimental and theoretical studies applied to the mechanism of [NiFe(Se)]-hydrogenases, definitive experimental evidence for the site of H_2 binding is lacking, though most models support Ni in this function [89,90]. Reaction intermediates isolated under catalytic conditions have been modeled into a mechanism of activation, but many of the details are unresolved [6,118,120,121].

Table 2
States identified in [NiFe(Se)]-hydrogenases under H_2.

Catalytic state	Oxidation state		EPR (g-values)	FTIR (cm^{-1})		Ligand
	Ni	Fe		νCO	νCN	
[a]Ni-SI	II	II	Silent	1943	2075, 2086	H_2O
Ni-C	III	II	2.21, 2.15, 2.01	1961	2074, 2085	H^-
Ni-R	II	II	Silent	1948	2061, 2074	H
[b]Ni-L	I	II	2.28, 2.11, 2.05	1911	2048, 2062	H^+

[a] Ni-TR for [NiFeSe] hydrogenases.
[b] Photoproduct observed after illumination at <200 K.

In the resting state of [NiFe(Se)]-hydrogenases, Ni-SI, the Fe center lies within H-bonding distance of the Ni, whereby H_2 binding and heterolytic cleavage at Ni^{II} would be facilitated by PT to result in a μ-hydride intermediate (Fig. 5) [122]. Alternatively, experimental and theoretical models propose initial H_2 binding and activation can occur at the Fe site, when the Ni is modeled as high spin Ni^{II} [120,121,123]. Cleavage is proposed to result in PT to one of the coordinating thiolate or selenate groups, and subsequently to nearby conserved Glu [116]. The resulting hydride is bound to the Ni-C (or Ni-R) intermediate and based on spectroscopic analysis together with density functional theory (DFT) calculations, it is proposed to adopt a bridging conformation between the Ni and Fe atoms [122,124,125]. Much of the detail on reaction intermediates of [NiFe]-hydrogenases has relied on studies of a subset of the [NiFe(Se)]-hydrogenases. Thus, it is quite possible for unique aspects to emerge from future studies of a more diverse array of enzymes.

3.2. [FeFe]-hydrogenases

3.2.1. Catalytic site structure and coordination sphere

Two x-ray crystal structures of [FeFe]-hydrogenases from *Clostridium pasteurianum* (*Cp*I) and *Desulfovibrio desulfuricans* (*Dd*H) revealed the active site H cluster (Fig. 4B), a unique [6Fe-6S] organometallic cluster comprised of a [4Fe–4S] subcluster and 2Fe subcluster, which are linked through a protein cysteine thiolate ligand [8,9,126]. The geometric and electronic properties of the 2Fe subcluster are closely connected to the [4Fe–4S] subcluster by strong spin coupling exchange through the Cys thiolate ligand between the two subclusters [127–132].

Reduction of protons to H_2 occurs at the 2Fe subcluster site, which includes a proximal Fe atom (Fe_p) and distal Fe atom (Fe_d) (in relation to the [4Fe–4S] cubane), each bound to a terminal CO and CN— ligand, bridging/semi-bridging CO ligand, and a bridging dithiolate ligand. A

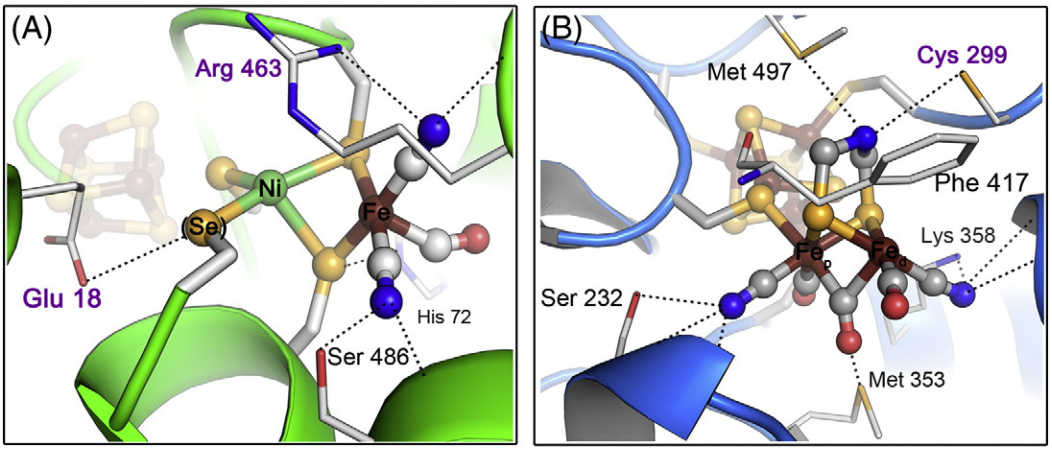

Fig. 4. X-ray structures of binuclear cofactors of the hydrogenase catalytic sites. (A) The NiFe cluster of [NiFe(Se)]-hydrogenases. (B) The 2Fe subcluster of the [FeFe]-hydrogenase H cluster. Images rendered from PDB codes 2FRV ([NiFe] hydrogenase) [266] and 3C8Y ([FeFe] hydrogenase *Cp*I from *Clostridium pasteurianum*).[267] The H-bonding interactions (dashed lines, black font) and conserved, exchangeable groups (dashed lines, purple font) are labeled. Ni, nickel; Se, selenium; Fe, iron.

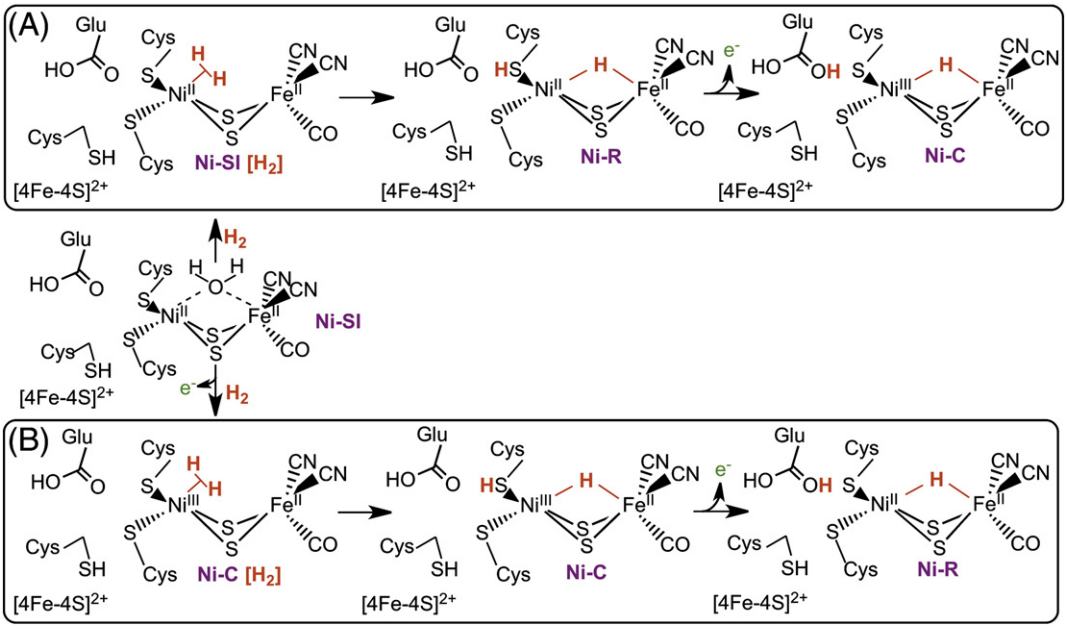

Fig. 5. Model of the H_2 binding, and the initial activation steps, catalyzed by [NiFe(Se)] hydrogenases. Scheme (A) proposed by Bruschi, M., et al. [120]. Scheme (B) is a summary of activation proposed by Wu and Hall [121]. Green signifies electron transfer reactions.

ligand exchangeable coordination site also resides at Fe_d and is a potential site for the formation of hydride intermediates [7]. The CO and CN⁻ ligands promote reversible heterolytic H_2 cleavage and stabilize low spin states of the two Fe atoms of the subcluster [10,133]. The CN⁻ ligands further fine-tune the energy levels of the frontier orbitals of the [4Fe–4S] and 2Fe subcluster moieties, which helps make possible fast electron transfer (ET) between the two subclusters during catalysis [134]. The functional bridgehead group of the dithiolate ligand was recently confirmed as an amine [135,136], an assignment previously suggested from analysis of *Dd*H [137] and advanced EPR methods [127], and theoretical models to function in PT to Fe_d during H_2 activation [138].

In the enzyme, the H cluster resides in a hydrophobic pocket where nearby residues participate in H-bonding interactions to the diatomic ligands of the 2Fe subcluster [8,126,139]. The surrounding protein framework finely-tunes the H-cluster and is considered to play an important role in regulating its catalytic activity, electronic properties, and potential hydride binding sites [14,23,130,140–143]. Within the catalytic site are several conserved, charged residues that form the secondary coordination sphere, with exchangeable groups proposed to function in the transfer of protons, water coordination, and bonding interactions with the H cluster (Fig. 4B) [7,14,23,139,144].

3.2.2. The emerging role of [4Fe-4S] in the [FeFe]-hydrogenase catalytic cycle

Recent spectroscopic investigations of the minimal algal [FeFe]-hydrogenase *Cr*HydA1, have put focus on the [4Fe-4S] and its role as an ET relay during catalysis (Fig. 6, and Table 3). Initial spectroelectrochemical FTIR and EPR investigations on *Dd*H led to identification of a "super-reduced" [149], or H_{sred}, H cluster state, which was later confirmed in *Cr*HydA1 and assigned as a $[4Fe–4S]^{1+}$–Fe^IFe^I [150,153]. Further EPR and FTIR studies of *Cr*HydA1 under reducing conditions provided evidence for multiple H cluster intermediates containing a reduced $[4Fe–4S]^{1+}$ during both catalytic H_2 activation and proton reduction. This implicated a role as an electron mediator between 2Fe and Fd [148,154]. X-ray spectroscopy along with DFT on *Cr*HydA1 found that for a complete model of the H-cluster, the LUMO resides over the [4Fe–4S] subcluster and is close in energy to the HOMO localized on Fe_d of 2Fe, further supporting its role as an initial relay into the 2Fe subcluster during H_2 activation [155].

These forms of H-cluster reduced states with $[4Fe–4S]^{1+}$ have led to revisions [148,150] of earlier models for H_2 activation by [FeFe]-hydrogenases [156]. Formally, H_2 activation was principally based on PT/ET transitions between the H_{ox} ($[4Fe–4S]^{2+}$–$Fe^{II}Fe^I$) and H_{red} ($[4Fe–4S]^{2+}$–Fe^IFe^I) states. These two redox states of the H cluster were assigned from early spectroscopic studies of more complex [FeFe]-hydrogenases containing additional FeS cofactors, or "F clusters" [128,129,157–160]. Thus, it is possible that trapping of $[4Fe–4S]^{1+}$ intermediates in these enzymes was prevented by ET between the H cluster and F clusters during H_2 activation. Alternatively, it is possible that some of these intermediates that have been observed in CrHydA1 might be unique to this enzyme due to its lack of F clusters.

The role of H_{sred} as a catalytic intermediate has come under question recently based on protein film voltammetry (PFV) studies of *Cr*HydA1. In one case, *Cr*HydA1 was observed to inactivate at low potentials, leading to the hypothesis that H_{sred} might not be catalytically relevant [149,161]. This observation differs from the high catalytic currents of enzymes at the low reduction potentials (<500 mV vs. NHE) [150,162,163], conditions that enrich for the H_{sred} state in spectroelectrochemical FTIR [152]. Clearly, identification of a $[4Fe–4S]^{1+}$ in *Cr*HydA1 under different reducing conditions indicates that oxidation of H_2 is a tightly coupled two-electron, two-proton reaction, and facile intramolecular ET steps to F clusters of more complex [FeFe] hydrogenases likely prevented the earlier detection of a $[4Fe–4S]^{1+}$ oxidation state. Mutants where PT is blocked or disrupted could be a useful strategy towards capturing reduced states and transient intermediates during catalysis [156].

3.2.3. H_2 binding and activation at the H cluster; activation at Fe_d^{II}

The resting state of the H clusters in *Cp*I, *Dd*H and *Cr*HydA1 are formally assigned to a mixed-valent 2Fe subcluster paired to a diamagnetic $[4Fe–4S]^{2+}$ subcluster based on the collective EPR/FTIR spectra of oxidized states (H_{ox}) of various enzymes (Table 2). Oxidation state assignments of the Fe_d/Fe_p pair in the H_{ox} state, and the localization of the unpaired spin, are currently under debate [127,132,164] (Fig. 6). Nevertheless, there is agreement that the initial step of H_2 binding occurs at the open coordination site of Fe_d in the Fe^{II} oxidation state. Evidence in support of this comes from spectroscopic studies on ^{57}Fe labeled *Dd*H poised in H_{ox} and CO-inhibited states [127]. The effect of CO on H_{ox} was used to interpret the effect of H_2 binding on the H cluster electronic state. CO binding induced stronger couplings between the [4Fe–4S] cubane and the 2Fe subcluster, consistent with a shift of the unpaired

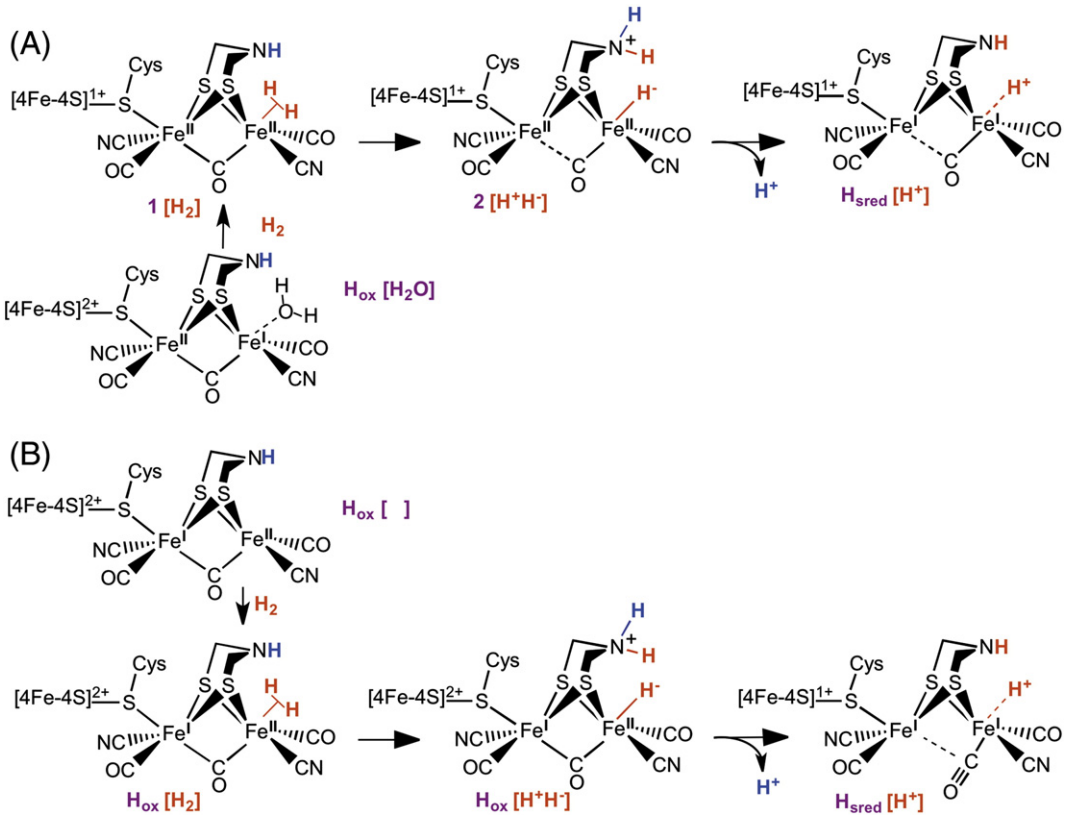

Fig. 6. Model for the H_2 binding and activation steps by [FeFe]-hydrogenases. Two alternative schemes are presented based on DFT and spectroscopic studies of [FeFe]-hydrogenases. Scheme (A), the resting H_{ox} state is assigned as a $[4Fe–4S]^{2+}$ subcluster and a 2Fe subcluster as Fe_p^{II}/Fe_d^{I} based on FTIR and EPR spectra and DFT calculations of oxidized *Cp*I and *Cr*HydA1 [132,146,148,149], and involves a diferrous 2Fe intermediate for H_2 binding and activation[166]. Scheme (B), the resting H_{ox} state is assigned as $[4Fe–4S]^{2+}$ and 2Fe as Fe_p^{I}/Fe_d^{II} [6,127,150,152], where H_2 binding and activation occurs on a mixed valent 2Fe at the Fe^{I} site. Green identifies intramolecular ET steps. The initial PT step of H_2 activation from Fe_d to the bridgehead amine ligand is shown in red.

spin from the 2Fe subcluster toward [4Fe–4S] (e.g., electron exchange from $Fe_d \rightarrow Fe_p$). More recently pulse EPR studies on isotopically labeled H cluster with $^{13}C^{15}N$ ligands support assignment of the unpaired spin in the H_{ox} state to Fe_d and delocalization of the spin over both Fe ions upon CO binding, a picture also consistent with a shift of unpaired spin toward [4Fe–4S] [164]. A completely synthetic H cluster mimic has also been shown to proceed via a $Fe^{I} \rightarrow Fe^{II}$ transition of a diiron/dithiolate subsite to accomplish H_2 binding and activation [165]. In this example of bio-inspired chemistry, the diferrous state of the diiron subsite was induced via oxidation by a covalently attached ferrocene acting as a *functional* mimic of the [4Fe–4S] subsite. The intramolecular ET step led to binding and activation of H_2 at the open coordination site of the Fe_d^{II} site, similar to the mechanism based on enzyme studies as proposed in Fig. 6, Scheme B [166].

3.3. Common functional themes

3.3.1. Catalytic bias and electron-transfer reactions

Under electrochemical PFV or biochemical assays with redox dyes of varying potentials, different hydrogenases have been shown to possess differences in catalytic bias, or reaction directionality [12,167,168]. The degree of reaction bias, or extent that H_2 production or oxidation is favored, is intrinsic to each particular hydrogenase [167,169–171]. This property has implications in the function of hydrogenase in a metabolic network, which is how the H_2 activation step is coupled to specific redox partners. Most or all members of each enzyme class have been shown by PFV to operate near to the thermodynamic potential of the $H_2/2H^+$ redox couple. Structural factors that control enzyme reaction bias, and to what degree these are enzyme specific, remain ongoing

Table 3
Redox states identified in [FeFe]-hydrogenases under H_2.

Catalytic state	Oxidation state			EPR (*g*-values)	FTIR (cm^{-1})		Fe_d Terminal Ligand
	2Fe		[4Fe-4S]				
	Fe_p	Fe_d			νCO	νCN	
[a]H_{ox}	II	I	2+	2.10, 2.04, 1.99	1964, 1940, 1802	2089, 2071	H_2O
[b]H_{ox}	I	II	2+	2.10, 2.04, 1.99	1964, 1940, 1800	2088, 2072	H_2O
[c]H_{red}	I	I	2+	Silent	1916, 1891, 1792	2038, 2034	H^+
[d]H_{red}	I	I	2+	Silent	1935, 1891, 1793	2083, 2070	Open (H)
[e]H_{red}	I	I	2+	Silent	1965, 1916, 1894	2079, 2041	H^+
[f]H_{sred}	I	I	1+	ND	1955, 1932, 1883	NA	NA
[g]H_{sred}	I	I	1+	2.08, 1.94, 1.88	1954, 1919, 1882	2070, 2026	H^+
[h]H_{sred}	I	I	1+	Broad 2.3–2.07	1933, 1883, ?	2085, ?	H^+

[a]Based on [145–149]. [b]Based on [127,150,151]. [c]Based on [148]. [d]Based on [23,150,152,153]. [e]Based on [10,149,150,152,153]. [f]Based on [149]. [g]Based on [150,152]. [h]Based on [148], "?" signifies unresolved assignments for CO/CN modes.

areas of investigation. Several hypotheses have been proposed for how bias might be controlled based on differences in the enzyme structures. One model for bias control is based on the differences in the potential of the electrons entering the catalytic site [170,172]. Thus, the evolution of enzyme and accessory cluster diversity has a significant role in tuning the catalytic function of the particular hydrogenase to couple to a given metabolic pathway. Other possible controlling factors of reaction bias are proton availability [23], midpoint potentials of the $1e^-$ oxidized/reduced forms of bimetallic clusters, role of secondary sphere coordination environment [173] and the accessibility of metal oxidation states and electronic structures [168].

3.3.2. Low spin, low-valent Fe(CO)(CN) centers and H_2 activation

A functional theme that has emerged from theoretical and model compound studies of hydrogenase catalytic sites is the role of a $Fe^{I/II}(CO)(CN)$ center in enzymes that function in H_2 binding and activation. The functional importance of the Fe site in [NiFe]-hydrogenases is perhaps less obvious than for the [FeFe]-hydrogenases where H_2 binding and activation occur directly at a Fe(CO)(CN) subsite. For both enzyme families, inclusion of this organometallic functional group as a component of the bimetallic catalytic sites is likely required to keep the respective metal centers in low-spin, low valence states, where the reaction coordinate for H_2 activation is thermodynamically favored [174–176].

4. Maturation

4.1. [NiFe]-hydrogenase active site assembly

Six *hyp* genes denoted *hypA–hypF* (and their associated homologs) are required for biosynthesis and insertion of the $NiFe(CN)_2CO$ catalytic cluster into the large subunit of [NiFe]-hydrogenases.

4.1.1. The synthesis of thiocyanate: HypE and HypF

HypC, D, E, and F proteins are involved in $Fe(CN)_2CO$ biosynthesis and are absolutely required for maturation of [NiFe]-hydrogenases [177–179]. The cyanide ligands of the active site cluster are derived from carbamoylphosphate in a series of reactions involving HypE and HypF (Fig. 7) [180]. HypF is a monomeric protein of ~82 kDa that utilizes carbamoylphosphate as substrate and first converts it to carbamate and then to carbamoyladenylate (in an ATP-dependent step) before finally transferring the carbamoyl functional group to the C-terminal cysteine residue of HypE (Fig. 7I–V) [180,181]. The intermediates that are produced by HypF are quite unstable, however, a mechanism for their formation is suggested by the HypE-HypF complex, a heterotetrameric structure comprised of a HypE dimer (monomeric size is ~35 kDa) flanked by two HypF molecules [182]. The structure supports a mechanism in which the N-terminal, HypF acylphosphatase domain hydrolyzes carbamoylphosphate to carbamate and inorganic phosphate; carbamate is transferred through an internal channel to the YrdC domain where ATP is bound and carbamoyladenylate formation occurs with release of pyrophosphate [182,183]. Carbamoyl group transfer to the C-terminal cysteine of HypE, with the liberation of AMP, occurs within the C-terminal Kae1-like domain [182]. The Kae1-like domain of HypF binds a mononuclear Fe ion via two His and two Asp, in similar fashion to other ASKHA superfamily members like Kae1 and TobZ [182, 184–186]. In HypF, this Fe ion coordinates the carbamoyladenylate moiety, promoting the transferase reaction [182].

The thiocarboxamide group bound to HypE's terminal Cys is converted to thiocyanate in a manner reminiscent of reactions catalyzed by formylglycinamide ribonucleotide amidotransferase (PurL) and aminoimidazole ribonucleotide synthetase (PurM); HypE, PurL, and PurM all utilize ATP to form phosphoryl anhydride intermediates [187–189]. In the case of HypE, the thiocarboxamide oxygen group is within van der Waals distance of the γ-phosphate group of ATP and is optimally positioned for in-line attack [190]. The thiocarboxamide

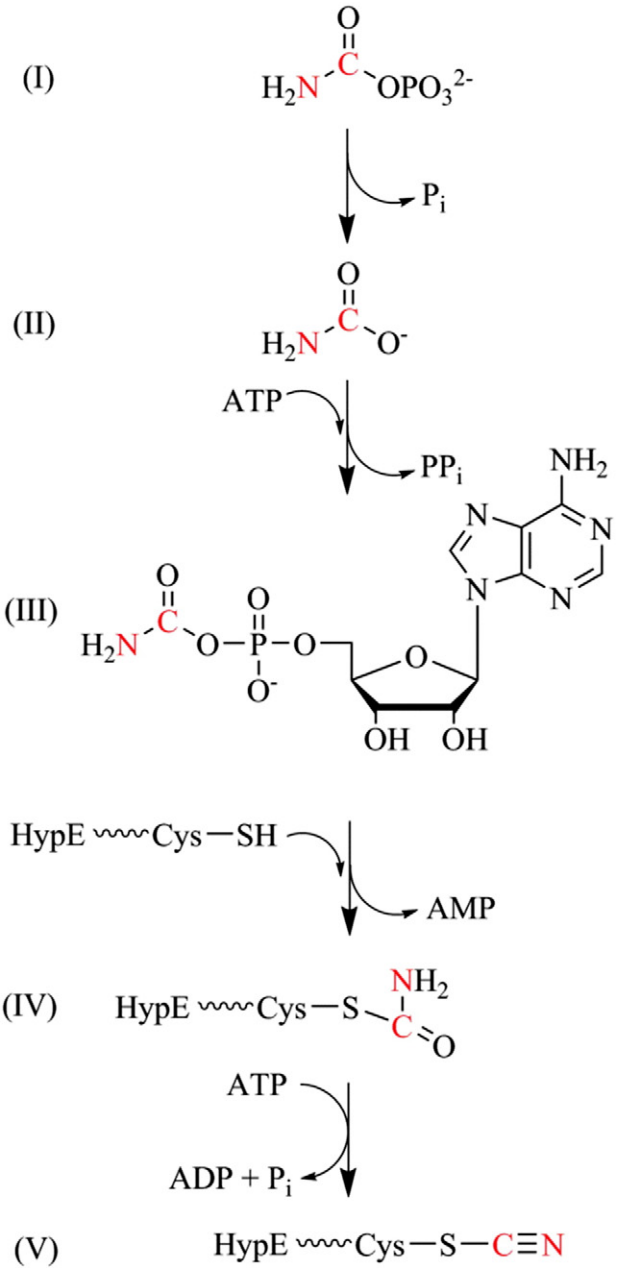

Fig. 7. Thiocyanate biosynthesis in [NiFe]-hydrogenase maturation. HypF acts on carbamoylphosphate (I), converting it first to carbamate (II), and then to carbamoyladenylate (III). The carbamoyl functional group is then mobilized to a cysteine on HypE's C-terminus (IV), where it is converted to thiocyanate (V).

group is proposed to become activated via deprotonation by an active site H_2O molecule. This H_2O hydrogen bonds to Lys134 which in turn is in close proximity to an Arg residue that decreases its effective pKa to approximately 5.1, allowing Lys134 to act as a general base [190]. Following the in-line attack of ATP, ADP is released and a thiocarbamic phosphoryl anhydride species is formed [189,190]. This iminophosphate intermediate is then activated for dephosphorylation via deprotonation of the imino nitrogen by a conserved Glu272 residue [190]. The resulting thiocyanate bound to Cys at the C-terminus of HypE is on a flexible loop that not only permits the insertion of the critical cysteine residue near the carbamoylation active site in the Kae1 domain of HypF, but then also facilitates delivery of the thiocyanate to the HypC-HypD complex where delivery of CN^- to iron occurs (Fig. 8) [180,189,191].

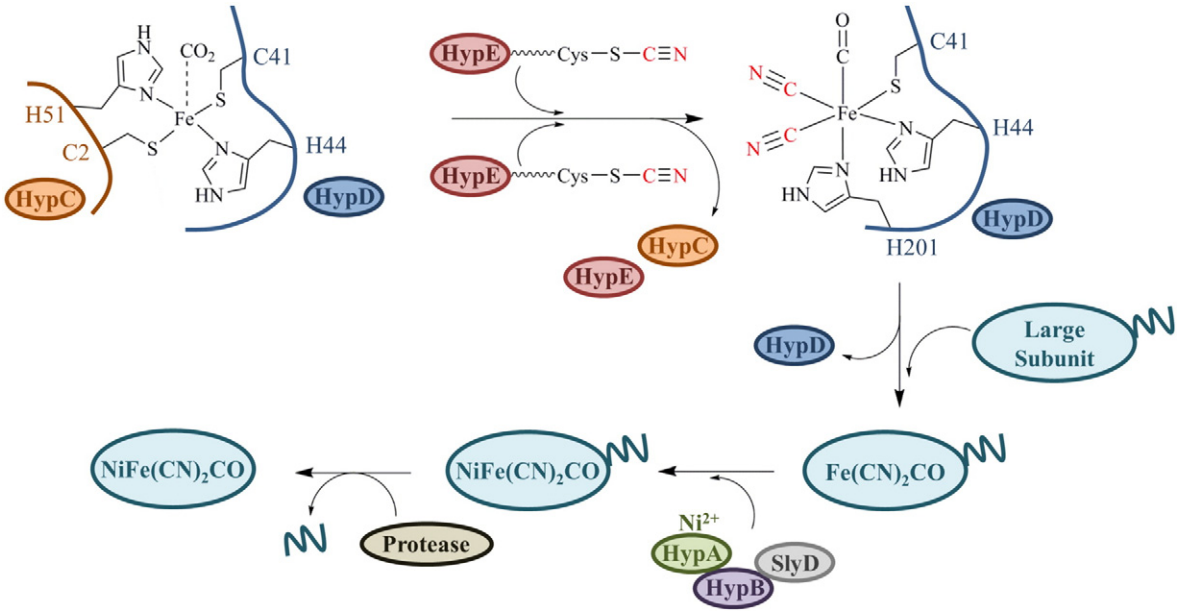

Fig. 8. $Fe(CN)_2CO$ biosynthesis and large subunit processing in [NiFe]-hydrogenase maturation. Thiocyanate delivery from HypE to the HypC–HypD complex results in HypC dissociation. The $Fe(CN)_2CO$ unit bound to HypD (*E. coli* amino acid numbering) is then transferred to the large, precursor subunit of [NiFe]-hydrogenase. While the source of CO is unresolved, recent biochemical evidence has been presented showing the association of CO_2 with HypC (see main text), suggesting that HypC delivers Fe–CO_2 to HypD where it is reduced to CO. Accordingly, CO_2 is shown here to be bound to the HypC–HypD complex. While HypC is known to associate with the apo-precursor form of the hydrogenase large subunit and has been proposed to be involved in iron mobilization to the hydrogenase [192–194], it is not shown to do so here as the addition of cyanide from HypE would effectively replace the ligand environment of the HypC–HypD complex that is provided by HypC; it is possible that following its dissociation at this stage, HypC associates with the apo-hydrogenase large subunit, fulfilling its role as a chaperone protein [193,195,202,203]. Following insertion of Ni^{2+}, a short C-terminal peptide is proteolytically processed, affording the mature [NiFe]-hydrogenase enzyme.

4.1.2. $Fe(CN)_2CO$ cofactor biosynthesis: HypC and HypD

Proteins in the HypC family of proteins are approximately 10 kDa in size and associate with HypD and a precursor form of the hydrogenase large subunit, and also form a ternary complex with HypD and HypE [36,192–197]. HypC is structurally simple, being comprised of a C-terminal α-helix joined to a β-barrel in what is known as an OB-fold [196,198,199]. HypD proteins are approximately 40 kDa in size and contain a unique C-terminal $CX_{14}CX_6CX_{16}C$ motif in domain III that coordinates a [4Fe–4S] cluster, whereas domains I and II are both characterized as Rossmann folds [194,199,200]. The cluster environment in domain III shares similarities with the ferredoxin:thioredoxin reductase system wherein a redox cascade is created that involves the [4Fe–4S] cluster and a pair of cysteine residues that are in close proximity to it, although these Cys residues are not absolutely conserved in all HypD isoforms [197,199,201]. The redox cascade is extended to four other conserved motifs in HypD (CGXHXH, GPGCPVCX_2P, GFETT, and PXHVSX_3G) and this conduit has been proposed to play a role in the mechanism of iron cyanation [195,196,199].

Co-expression studies revealed that substoichiometric amounts of HypE were associated with a HypC–HypD complex and that this complex accepted CN^- from HypE; CN^- was not transferred to either HypC or HypD when they were expressed singly, suggesting that the HypC–HypD complex shared coordination of the CN^- group (Fig. 8) [194]. The exact details of diatomic ligand coordination to Fe at this stage of maturation are not fully resolved and a consensus model has not yet been attained. Structural characterization of the HypC–D–E ternary complex provides a picture for how the conserved motifs of HypD create the scaffold for Fe-cyanation; a model is proposed wherein Cys38 from HypD and Cys2 of HypC (*Thermococcus kodakarensis* numbering) bind an Fe ion along with two CN^- ligands and two unresolved moieties [196]. Spectroscopic characterization of anaerobic preparations of HypC–HypD complexes containing substoichiometric amounts of HypE from *E. coli* [195] and HypC–HypD complexes from both *Ralstonia eutropha* and *E. coli* [197] show FTIR bands that correspond to an $Fe(CN)_2CO$ species similar to that observed in [NiFe]-hydrogenase. Importantly, the characterization of the HypC–HypD complex reported by Soboh et al. revealed the existence of two labile Fe ions in addition to the [4Fe–4S] cluster, and also showed an IR feature at 2337 cm^{-1} assigned to νCO_2; Cys41 in HypD was shown to be required for the coordination of CN^-, CO, and CO_2, presumably to one of the labile Fe ions [195]. Analysis of anaerobically purified HypD showed that it contained FTIR bands associated with a CO and two CN^- groups, consistent with the presence of an $Fe(CN)_2CO$ moiety; no 2337 cm^{-1} CO_2 feature was present (Fig. 8) [202].

Insight into the significance of CO_2 was provided in a subsequent report that examined individually purified HypC along with its *E. coli* homolog HybG [203]. Characterization revealed that under anaerobic conditions the proteins contained ~0.3 mol Fe per mol protein and exhibited single FTIR bands arising from CO_2. Evaluation of variant HypC proteins conclusively demonstrated that both Cys2 and His51 were absolutely required for Fe and CO_2 coordination [203]. This observation coupled with the presence of the 2337 cm^{-1} feature in the HypC–HypD complex suggests that HypC delivers Fe–CO_2 to HypD where the CO_2 is reduced to CO (Fig. 8) [195,203]. Importantly, these results and others suggest that metabolic CO_2 is the source of the CO ligand, although this has yet to be experimentally demonstrated [203,204].

In the case of HypD, Cys41, Cys69, and Cys72 are all critical for synthesis of the intermediate $Fe(CN)_2CO$ cluster [202]. Collectively, these data suggest a model wherein Cys2 and His51 of HypC and Cys41 and His44 of HypD (*E. coli* numbering) come together to coordinate a precursor Fe ion in a tetrahedral environment; addition of the CO and the CN^- ligands results in HypC dissociation and addition of His201 to accomplish formation of the octahedrally coordinated $Fe(CN)_2CO$ unit on HypD (Fig. 8) [202,203]. The assignment of His44 and His201 as the fourth and fifth ligands is not established and it is also possible that Cys69 and Cys72 perform this function [202].

4.1.3. Insertion of Ni^{2+}: HypA and HypB

Two of the *hyp* gene products, HypA and HypB, are involved in acquiring and inserting Ni^{2+} and studies have shown that these proteins are not absolutely required because addition of Ni^{2+} during anaerobic growth restores hydrogenase activity in mutant cell lines [178,179,205,206]. The insertion of Ni^{2+} into the large subunit occurs only after

the $Fe(CN)_2CO$ moiety is present (Fig. 8) [36,207]. HypA is a 140 amino acid protein that exists in both monomeric and dimeric states [208]. HypA coordinates a single Ni^{2+} ion with micromolar affinity via an N-terminal MHE motif [208–210], and also binds Zn^{2+} through the cysteine thiolates of a zinc finger motif [208]. While the Ni^{2+} and Zn^{2+} binding domains are independent of one another, the presence of Ni^{2+} appears to help dictate the orientation of these two domains, likely as a mechanism to mediate protein-protein interactions [201,208,210]. Moreover, HypA exhibits low sequence conservation outside of the Ni^{2+} and Zn^{2+} motifs; this mirrors the sequence diversity in both HypB and [NiFe]-hydrogenase, all three of which putatively interact during Ni^{2+} delivery [201].

HypB is a Ni^{2+}-metallochaperone that has GTPase activity, which is essential to achieve complete hydrogenase maturation [36,211,212]. Several HypB homologs exist with varying sequence-based metal binding properties. For example, *E. coli* HypB contains a high affinity, Ni^{2+} binding N-terminal CXXCGC motif (which is not absolutely conserved) and a C-terminal GTPase domain that can bind either Ni^{2+} or Zn^{2+} [213–215]. HypB dimerization is Ni^{2+}-dependent and its GTPase activity is modulated by metal binding [216]. The structure of the nucleotide bound *Methanocaldococcus jannaschii* enzyme shows two Zn^{2+} ions bound at the dimer interface, utilizing a combination of cysteine thiolate, histidine imidazole, and H_2O as ligands [214]. Structural analysis of the nucleotide bound form of *Helicobacter pylori* HypB, on the other hand, reveals a single Ni^{2+} ion bound at the dimer interface via the tetrathiolate coordination of Cys106 and Cys142 from each monomer, whereas in the absence of nucleotide His107 becomes a metal ligand [217]. Cys142 is part of the GTPase Switch II motif and is likely a ligand to both Ni^{2+} and Zn^{2+} bound forms of HypB regardless of the nucleotide bound state [217]. This enables Cys142 to couple GTP hydrolysis with metal binding and delivery, potentially providing a mechanism wherein HypB distinguishes between Ni^{2+}- and Zn^{2+}-loaded forms, precluding delivery of zinc to the large subunit during maturation [214,217,218].

While the molecular mechanism for Ni^{2+} transfer to the large catalytic subunit is not yet fully resolved, delivery of nickel *in vivo* is likely modulated by protein-protein interactions and evidence for HypA, HypB, SlyD (a Ni^{2+} metallochaperone in *E. coli*), and HycE (the large subunit of hydrogenase 3 in *E. coli*) complexes have been reported (Fig. 8) [219–224]. HypB's interaction with the large subunit requires the presence of HypA, providing support for HypA being the docking protein between HypB and HycE in the nickel delivery step [210,219,220]. Moreover, HypA selectively removes Ni^{2+} from the GTPase domain of HypB and this metal release appears to be stimulated by GTP hydrolysis [225]. Despite the observation that Ni^{2+} binding to HypB partially inhibits the GTPase activity of HypB [217], it is presumed that complex formation *in vivo* alleviates this retardation and promotes nickel mobilization [225]. Along these lines, it has been demonstrated that in *E. coli*, SlyD delivers Ni^{2+} to HypB and heterodimer formation between these proteins promotes GTP hydrolysis by HypB [226]. This provides a mechanism whereby SlyD acts to mediate the delivery of Ni^{2+} to the large subunit via the GTPase activity of HypB; the favorable interaction between SlyD and the GDP-bound form of HypB may help drive the Ni^{2+} insertion process and overcome any thermodynamic barriers associated with metal ion delivery [215,221,226].

4.1.4. Ni^{2+} dependent proteolysis and active site closure

In a final step to accomplish maturation of the large catalytic subunit, a peptide on the C-terminus is processed (Fig. 8). Peptide length varies somewhat among different hydrogenases; a peptide of approximately 15 residues is most commonly cleaved although maturation of *E. coli* hydrogenase 3 results in the removal of 32 residues [191]. The peptide extension of the large subunit is necessary for interaction with HypC, and its presence helps keep the large subunit in an open conformation for both Ni^{2+} and $Fe(CN)_2CO$ insertion [192,193,227]. The endopeptidases in *E. coli* that mature the three cognate [NiFe]-hydrogenases are HyaD (hydrogenase 1), HybD (hydrogenase 2), and HycI (hydrogenase 3) [228]. These enzymes recognize the DPCXXCXXH/R consensus motif that helps coordinate the active site metal center in [NiFe]-hydrogenase; the proteases cleave between the basic His/Arg and the nonpolar Met/Ile/Val/Ala residues [229–232]. Nickel promotes the recognition of the binding motif by the proteases and explains why cleavage only occurs after Ni^{2+} has been inserted [233]. Structural characterization of HycI suggests that two conserved Asp residues may be critical for Ni^{2+} coordination, implying that HycI functions via metal-based activation wherein the Ni^{2+} ion polarizes the carbonyl oxygen of the peptide bond, which in turn fosters hydrolysis by H_2O [191,234]. Upon cleavage of the C-terminal peptide, a conformational change in the active site environment is induced which effectively internalizes the $NiFe(CN)_2CO$ moiety and affords the active enzyme [36].

4.2. [FeFe]-hydrogenase active site assembly

4.2.1. Identification of the maturation machinery

Three genes denoted *hydE*, *hydF*, and *hydG* are found in all organisms expressing [FeFe]-hydrogenase (HydA) and the expression of active HydA in *E. coli* requires coexpression with HydE, HydF, and HydG [40,235]. Activation of HydA expressed singly ($HydA^{\Delta EFG}$) is accomplished via addition of *E. coli* lysate mixtures containing HydE, HydF, and HydG together; $H_2(g)$ generation under these experimental conditions does not require exogenous small molecules, leading to the conclusion that the maturase enzymes utilize ubiquitous small molecules present in the cellular matrix to assemble the H-cluster [38,235]. Sequence annotation coupled with preliminary biochemical characterization demonstrated that HydE and HydG both belong to the radical *S*-adenosyl-L-methionine (SAM) enzyme superfamily, while HydF is an FeS cluster binding GTPase [40,235–237]. Point mutations in the radical SAM motifs of HydE and HydG, as well as the FeS cluster and GTPase regions of HydF, all proved to be deleterious to achieving $HydA^{\Delta EFG}$ activation, meaning that HydE, HydF, and HydG functionalities are indispensable for proper H-cluster synthesis [235].

4.2.2. Activation of $HydA^{\Delta EFG}$ requires a preformed [4Fe–4S] cluster

Spectroscopic and structural characterization of the monomeric [FeFe]-hydrogenase I of the green algae *Chlamydomonas reinhardtii* (*Cr*HydA1) has provided important insights into the maturation process. The enzyme $CrHydA1^{\Delta EFG}$, obtained from a strain that lacks the genes encoding the three maturation proteins HydE, HydF, and HydG, contains the [4Fe–4S] cubane of the H-cluster [32,238]. Importantly, $CrHydA1^{\Delta EFG}$ is readily activated by *E. coli* lysate containing HydE, HydF, and HydG. Moreover, the metal-free form of the protein must first be chemically reconstituted with iron and sulfide prior to successful activation by lysate [238]. These results show that activation by the maturase enzymes requires a preformed [4Fe–4S] cluster that is presumably synthesized by the endogenous iron sulfur cluster assembly machinery of the cell, and also suggest that HydE, HydF, and HydG come together to synthesize the 2Fe subcluster component of the H-cluster [32,238]. *In vitro* labeling studies provide additional support for this model, as activation of $CrHydA1^{\Delta EFG}$ by ^{57}Fe-labeled HydE, HydF, and HydG lysates demonstrate the incorporation of ^{57}Fe into only the 2Fe subcluster of the H-cluster [239].

Comparison of the $CrHydA1^{\Delta EFG}$ structure to that of *Cp*I HydA provides insight into 2Fe subcluster insertion [32]. An electropositive channel filled with H_2O molecules runs from the protein surface to the active site cavity of $CrHydA1^{\Delta EFG}$; this channel is absent in the mature *Cp*I HydA structure, which contains two ordered loop regions that effectively shield the H-cluster from solvent. The loop regions in $CrHydA1^{\Delta EFG}$ are splayed open, providing clear access to the active site cavity for 2Fe subcluster insertion, which may either be electrostatically- or entropically-driven [32,139,240].

4.2.3. HydF is an iron sulfur cluster binding GTPase

HydF is a 47 kDa monomeric protein that binds FeS clusters and hydrolyzes GTP to GDP [237,241]. The purified state of this protein is a composite mixture of dimers and tetramers and structural characterization of the metal-free enzyme has suggested that FeS cluster(s) bind at subunit interfaces [242]. Both FeS- and GTP-binding motifs are essential for maturation in the *Ca* system [235]. Moreover, among the three maturase enzymes, only purified HydF when expressed in a genetic background of HydE and HydG ($HydF^{EG}$) could activate $HydA^{\Delta EFG}$. This activation was achieved in the absence of exogenously added proteins or small molecules, suggesting that HydF bound a precursor form of the H-cluster needed to achieve maturation [243].

Spectroscopic comparison of $HydF^{EG}$ to $HydF^{\Delta EG}$ revealed that both proteins bound $[4Fe\text{–}4S]^{+}$ clusters, but $HydF^{\Delta EG}$ samples contained additional LMCT features in UV-Vis spectra and an overlapping axial component in EPR spectra that are thought to arise from a $[2Fe\text{-}2S]^{+}$ cluster [241,243]. Preparations of *Ca* $HydF^{EG}$ revealed the existence of Fe–CN^{-}, Fe–CO, and Fe–CO–Fe species via FTIR spectroscopy [241,244]. These moieties were absent in $HydF^{\Delta EG}$, suggesting that HydE and HydG modified a [2Fe–2S] cluster precursor bound to $HydF^{\Delta EG}$ to produce the 2Fe subcluster of the H-cluster [41,241].

The FeS cluster states of both wild type and variant forms of HydF have been examined from various species and all of the results provide evidence for [4Fe–4S] cluster binding to HydF [237,241,244–249]. The coordination environment for the [4Fe–4S] cluster utilizes three conserved cysteine residues; the fourth coordination site appears to be variable, either being a histidine in the case of *Ca* HydF or a labile/exchangeable ligand in *Thermotoga maritima* (*Tm*) and *Thermotoga neopolitana* (*Tn*) HydF [242,246,248–250]. The observation and significance of $[2Fe\text{–}2S]^{+}$ cluster binding by HydF is still an open issue. While similar overlapping axial signals to those initially reported [241] have now been observed in reduced preparations of both *Ca* and *Tn* samples, it has been suggested this signal arises from a radical in close proximity to the $[4Fe\text{–}4S]^{+}$ cluster [248,249,251]. Regardless, multiple lines of evidence support the notion that the 2Fe subcluster bound by HydF resembles the H-cluster, and may potentially even be directly linked to the [4Fe–4S] cubane present on HydF [245,251].

While the exact role of GTP hydrolysis in [FeFe]-hydrogenase maturation remains unresolved, it is clear that the GTPase activity of HydF is not associated with the activation step of $HydA^{\Delta EFG}$ by $HydF^{EG}$ [241]. In a manner reminiscent of HypB [217], the GTPase activity of HydF is gated by different monovalent cations and these regulate GTP hydrolysis by ~40-fold. HydF therefore belongs to a subclass of GTPase enzymes wherein an alkali metal substitutes for the "arginine finger" of the partner GTPase activating protein [241]. The ability of $HydF^{EG}$ to mature $HydA^{\Delta EFG}$ is unaltered by HydF being in either a "GTPase-on" or a "GTPase-off" state, as established by accomplishing the activation in various alkali containing buffers [241]. However, it was observed that the rate of GTP hydrolysis was stimulated when HydF was assayed in the presence of either HydE or HydG, leading to the hypothesis that the production of GDP could be coupled to gating the protein–protein interactions associated with 2Fe subcluster assembly [241]. Experiments designed to probe protein–protein interactions between the maturases revealed that HydE and HydG do not bind to HydF concurrently, suggesting that the radical SAM enzymes associate with the same binding site on HydF [252]. Moreover, HydE and HydF bind to one another with an order of magnitude higher affinity than HydG binds to HydF and addition of GTP during dissociative phases causes enhanced detachment rates for both HydE–HydF and HydG–HydF complexes. [252]. Collectively, these results implicate GTP binding and hydrolysis as providing a mechanism for gating the interactions between HydE and HydG with the scaffold/carrier HydF, and this may be linked to nucleotide dependent structural changes in HydF.

While the majority of reports provide support for the indispensable nature of HydF during maturation [38,40,235,241,243,244], results with *Shewanella oneidensis* maturases suggest that HydF is nonessential. Specifically, the *in vitro Cp*I $HydA^{\Delta EFG}$ activation studies with this system concluded that HydG is the only maturase enzyme absolutely required [247]. Interestingly, $HydA^{\Delta EFG}$ activation experiments using compounds designed to mimic the 2Fe subcluster show that these analogs can not only be loaded into a [4Fe–4S] cluster containing form of *Tm* HydF, but that this charged HydF can then fully mature $HydA^{\Delta EFG}$ [135]. A subsequent study demonstrated that the 2Fe biomimetic analogs could load into $HydA^{\Delta EFG}$ in the absence of HydF, showing that HydF is not required for *in vitro* activation [136].

4.2.4. HydG and diatomic ligand biosynthesis from tyrosine

HydG contains a CX_3CX_2C N-terminal motif identifying it as a member of the radical SAM superfamily of enzymes [40,253]. This 55 kDa monomeric protein exhibits high similarity to ThiH, an enzyme involved in thiamine pyrophosphate synthesis that cleaves L-tyrosine into *p*-cresol and dehydroglycine (DHG) [253,254]. Like ThiH, HydG also utilizes L-tyrosine as substrate and forms *p*-cresol (Fig. 9) [255]. HydG is differentiated from ThiH, however, in the existence of a 90 amino acid C-terminal extension containing a $CX_2CX_{22}C$ motif that harbors an accessory [4Fe–4S] cluster. The presence of the C-terminal [4Fe–4S] cluster is essential for [FeFe]-hydrogenase maturation, and the formation of the diatomic products CO and CN^{-} [37,164,235,256]. Diatomic ligand production during HydG catalysis was demonstrated both by derivatizing CN^{-} into a 1-cyanobenz[f]isoindole adduct [37] and by trapping CO via binding to deoxyhemoglobin [256]. Both of these diatomic species formed on the same time scale and it was hypothesized that they could be formed in a single step via a decarbonylation reaction involving DHG [37].

Analysis of HydG variant proteins demonstrated the absolute requirement of the C-terminal [4Fe–4S] cluster for formation of CO, although CN^{-} was still detected in variants containing either single or double point mutations to residues in the $CX_2CX_{22}C$ motif [257,258]. Assessment of a variant in which the three cysteines of the CX_3CX_2C N-terminal motif were altered to alanine revealed that SAM coordination to the enzyme occurs exclusively at the N-terminal cluster, despite the apparent site-differentiated nature of the C-terminal [4Fe–4S] cluster [258]. Evaluation of ΔCTD HydG, a variant that is missing the C-terminal domain, shows that the enzyme still acts as a tyrosine lyase [257–259]. Collectively, the results show that SAM binding and cleavage, with subsequent H-atom abstraction from tyrosine, and the generation of *p*-cresol and DHG, all occur within the core TIM barrel fold [251,258].

Spectroscopic characterization of intermediate species formed during catalysis provided direct evidence for heterolytic Cα–Cβ tyrosine bond cleavage, with generation of a *p*-cresolate radical and DHG (Fig. 9) [260]. Stopped-flow FTIR studies were used to monitor the fate of DHG to formation of either Fe–CO–CN or Fe–$(CO)_2$CN moieties. It was assumed that these species formed at the site differentiated Fe of the accessory [4Fe–4S] cluster although there is no direct experimental evidence for this [164]. In separate experiments, ^{57}Fe was used to track iron transfer from HydG to HydA, suggesting that the C-terminal [4Fe–4S] cluster in HydG becomes cannibalized to form the Fe–CO–CN building blocks of the 2Fe subcluster [164]. Furthermore, it is known that all five diatomic ligands of the H-cluster are derived from tyrosine [261], suggesting that multiple rounds of HydG catalysis are necessary for 2Fe subcluster formation. The mechanistic details of this process are unknown but must be resolved to delineate the role of HydF as a scaffold/carrier protein during biosynthesis [251].

4.2.5. An undefined role in H-cluster biosynthesis: HydE

In contrast to the extensive experimental data supporting the proposed roles of HydF and HydG in H-cluster biosynthesis, the role of HydE, a member of the radical SAM superfamily of enzymes, is still unknown. It has been suggested that HydE functions in an observatory role during maturation, possibly acting as a chaperone and assisting HydF during translocation of the 2Fe subcluster species [247]. It seems

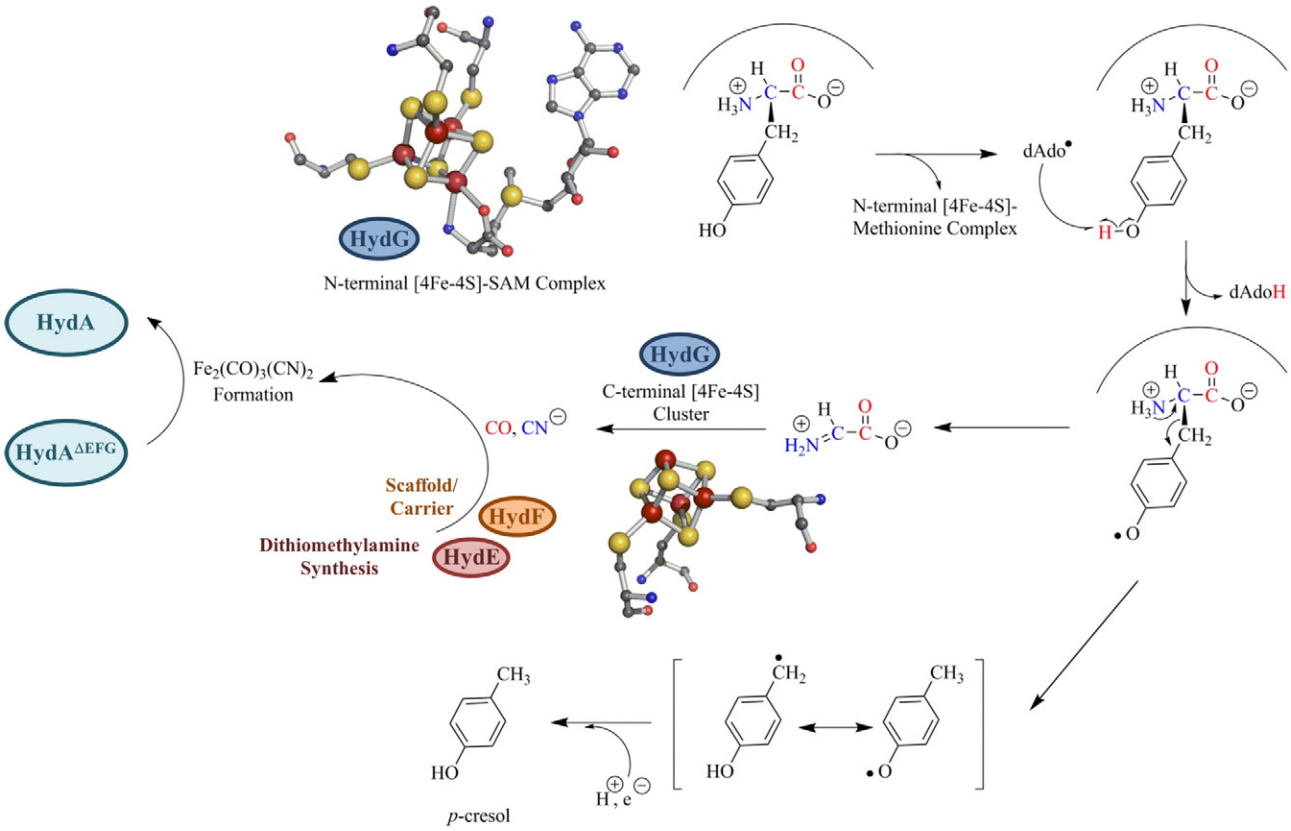

Fig. 9. Maturation scheme detailing hypothetical 2Fe subcluster biosynthesis in [FeFe]-hydrogenase maturation. HydG reductively cleaves SAM into methionine and a 5′-deoxyadenosyl radical species; following H-atom abstraction from the *para* phenolic position, tyrosine undergoes heterolytic Cα — Cβ bond cleavage to generate a *p*-cresolate radical and dehydroglycine. CO and CN^- are subsequently formed from dehydroglycine by an unknown mechanism involving the site-differentiated, C-terminal [4Fe-4S] cluster of HydG. Stopped-flow FTIR studies have shown the existence of Fe–CO–CN species that form during HydG catalysis (see main text), leading to the hypothesis that the C-terminal cluster is cannibalized during biosynthesis. Maturation of the 2Fe subcluster is completed via dithiomethylamine synthesis by HydE. HydF acts as a scaffold or carrier protein during the assembly process and transfers the 2Fe subcluster to $HydA^{\Delta EFG}$ to achieve [FeFe]-hydrogenase activation.

much more likely, however, that HydE utilizes a common metabolite and synthesizes the bridging dithiomethylamine ligand (Fig. 9). Evidence in support of this comes from the absolute requirement of HydE to achieve [FeFe]-hydrogenase activity in *E. coli* lysate experiments [38,40,235,238] and the difficult chemistries often associated with radical SAM enzymes [253]. HydE is a 42 kDa monomer and contains a CX_3CX_2C N-terminal motif, like other radical SAM enzymes [40,253]. However, it shows high sequence similarity to the methylornithine synthase PylB [236,251,262], rather than to enzymes like BioB that catalyze a sulfur insertion reaction [263]. While the significance of HydE's relationship to PylB is difficult to currently gauge, it is possible that it is an indication that these enzymes exhibit mechanistic parallels.

Multiple X-ray crystal structures of HydE exist and these include SAM and 5′-deoxyadenosine/methionine bound states [263–265]. The global architecture of HydE shows the existence of an internal electropositive cavity spanning the breadth of the $(\beta\alpha)_8$ TIM barrel fold. Three anion binding sites were observed within the internal cavity and SCN^- also binds here, potentially representing a pathway whereby a small molecule product(s) traverses from the top to the bottom of the barrel where delivery to a maturation partner protein occurs [263]. Surface plasmon resonance experiments indicate that HydE and HydF bind to one another with high affinity and even exist as a fused gene product in some organisms [40,252]. Regardless of these experimental findings, the substrate and mechanism of HydE in H-cluster biosynthesis remain unresolved. Although it is somewhat tenuous to assign it a role in dithiomethylamine synthesis, this seems the most likely given the precedence in the literature for the chemistries associated with HydF and HydG [251]. An intriguing aspect of HydE's presumed chemistry relates to the source of the sulfurs in the 2Fe subcluster. Future work will hopefully resolve if the sulfurs are derived from HydE's substrate or if they are cannibalized from HydG's C-terminal cluster.

4.2.6. Mechanistic parallels and differences between [NiFe]- and [FeFe]-hydrogenase biosynthesis

[NiFe]- and [FeFe]-hydrogenases are a superb example of convergent evolution and it follows that their biosynthetic pathways exhibit both commonalities and differences. Generally, it is straightforward to see that both active site assembly pathways require multiple maturation proteins involved in intricate chemical transformations. Moreover, each system utilizes a key scaffold protein that belongs to the same subclass of GTPase enzymes, which are activated by alkali metals. The formation and transfer of Fe-diatomic species among proteins is also an intriguing aspect shared by these systems, despite the fact that the source of these diatomic ligands is disparate. [FeFe]-hydrogenase assembly requires the involvement of two radical SAM enzymes, a feature that noticeably distinguishes it from [NiFe]-hydrogenase maturation.

5. Future frontiers of hydrogenase research

Hydrogenases are unique enzymes and the recent insights into their diversity, mechanism, and maturation have revealed many surprises that represent highly unique aspects of bioinorganic chemistry, as summarized here. Although a number of questions have been answered through the surge of recent work in this area, these exciting new discoveries also serve to open the door for perhaps more lines of inquiry than they have closed. It is likely that hydrogenases will have a central role in

clarifying the newly discovered mechanism of energy conservation termed electron bifurcation ([48]). In many systems these bifurcating reactions are likely key points for controlling and balancing electron flow in metabolism and a better fundamental understanding of these processes could potentially provide the basis for proactively controlling electron flow for the production of biofuels in engineered systems.

The striking functional diversity of [FeFe]-hydrogenases and [NiFe]-hydrogenases has interesting evolutionary implications and the further characterization of representatives of the various diverse classes of these enzymes will lead to more fascinating evolutionary insights. Most would agree that aspects of hydrogen metabolism must have been a component of the metabolism of early life and this appears to be supported for the [NiFe]-hydrogenases but arguably not for the [FeFe]-hydrogenases. The traditional dogma and natural impetus is that hydrogenase must be ancient or even must be primordial because of the perceived importance of hydrogen metabolism for early life. However, in the case of [FeFe]-hydrogense, the available data including their taxonomic occurrence among extant organisms is inconsistent with an ancient origin. This begs the question of whether [NiFe]-hydrogenases and [FeFe]-hydrogenases are both ancient, or primordial, and what the selective pressures might have been to independently evolve so-called functionally redundant enzymes. Further analysis of hydrogenase diversity in the context of their modes of metabolism could provide important new clues into hydrogenase ancestry as well as the ancestry of chemiosmosis and respiration. One driving force will likely be the close evolutionary relationship between one specific type of [NiFe]-hydrogenase (the group 4 energy-conserving enzymes) and the ubiquitous complex I of the aerobic respiratory chain. How did the latter evolve from a presumably H_2-metabolizing ancestor?

Additional insight gained into the relationship between sequence variations and the respective physiological roles for hydrogenases have potential to further identify the key structural determinants for catalysis. Hydrogenases are intriguing models for proton coupled electron transfer reactions and these are in some regards very special enzymatic reactions. We are taught in general chemistry of the separation between thermodynamics and kinetics in chemical reactions and that catalysts by definition exert the influence solely on the rates of reactions and don't influence whether a reaction is thermodynamically favorable. However for enzymes involved in proton coupled electron transfer reactions there is clear evidence that the properties of the enzymes themselves can influence the local concentration of substrates and/or products of a reaction and thereby its thermodynamic equilibrium. The chemical character and availability of proton donors and acceptor groups in the active site and the oxidation-reduction potential of active site metal and accessory clusters can essentially tune the concentration of protons and electrons and influence the equilibrium of hydrogenase reactions. The extent of this influence is not well understood and the simplicity of the hydrogenase reaction makes this an ideal model system for delineating the degree of this control, potentially significantly impacting biotechnology particularly in the area of biofuel production.

For [NiFe]- and [FeFe]-hydrogenases, as detailed in this review, there have been significant advances in our understanding of nonprotein ligand biosynthesis and protein maturation. The fact that diatomic ligands are generated from different sources and by different enzymatic reactions is intriguing given the unique nature of these ligands in biology. Why two independent mechanisms evolved to generate these ligands is unknown. This could be related to the physiology of their respective evolutionary ancestral organisms or the differences in the structures of the active site cluster and the requirement for different ratios of CO and CN^- for each class. There is, however, still much more work to be done. The illumination of the pathways for nonprotein ligand biosynthesis can be linked to a classic metabolic pathway elucidation problem in which the substrates are not defined and as such present significant challenges to researchers. Advances over the last decade have succeeded in identifying the substrates for carbon monoxide and cyanide synthesis but there are still a lot of mechanistic details that need to be resolved. For the [NiFe]-hydrogenase diatomic ligand biosynthesis, the existence of Fe–CO_2 FTIR bands both in HypC alone and when HypC is in a complex with HypD has led to the recent hypothesis that HypC delivers an Fe–CO_2 group to HypD where the CO_2 is reduced to CO. Experimental validation is needed, both for the transfer and delivery of this species from HypC to HypD, as well as for the source of the CO ligand being CO_2. For [FeFe]–hydrogenase diatomic ligand biosynthesis the presence of Fe–CO/CN species which form during HydG catalysis leads to the hypothesis that the C-terminal cluster is cannibalized in this process and that the iron-diatomic group(s) are then mobilized to HydF. Clearly for [FeFe]-hydrogenase H cluster biosynthesis, as described herein, there are differing perspectives on aspects of the mechanism of H cluster assembly. Some of this likely reflects differences in experimental designs, in particular how the H cluster maturation proteins are expressed and analyzed. It will be the challenge of the next generation of maturation studies to delineate the relevant insights being gleaned for all the expected approaches in the context of physiological or in vivo assembly.

The culmination of the new insights into hydrogenase diversity, mechanism, and maturation presented herein should have one convinced of the amazing qualities of hydrogenases as complex iron sulfur enzymes that span evolutionary time and which played a key role in the physiological diversification of life. These enzymes provide a one stop model system for studies of the evolution of early life processes, control of biological electron flow in metabolism, proton coupled electron transfer reactions in biological systems, complex metal cluster assembly, and the origin of modern day aerobic respiration.

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