Review

Constanze Pinske and R. Gary Sawers*

The importance of iron in the biosynthesis and assembly of [NiFe]-hydrogenases

Abstract: [NiFe]-hydrogenases (Hyd) are redox-active metalloenzymes that catalyze the reversible oxidation of molecular hydrogen to protons and electrons. These enzymes are frequently heterodimeric and have a unique bimetallic active site in their catalytic large subunit and possess a complement of iron sulfur (Fe-S) clusters for electron transfer in the small subunit. Depending on environmental and metabolic requirements, the Fe-S cluster relay shows considerable variation among the Hyd, even employing high potential [4Fe-3S] clusters for improved oxygen tolerance. The general iron sulfur cluster (Isc) machinery is required for small subunit maturation, possibly providing standard [4Fe-4S], which are then modified as required *in situ*. The [NiFe] cofactor in the active site also has an iron ion to which one CO and two CN^- diatomic ligands are attached. Specific accessory proteins synthesize these ligands and insert the cofactor into the apo-hydrogenase large subunit. Carbamoyl phosphate is the precursor of the CN^- ligands, and recent experimental evidence suggests that endogenously generated CO_2 might be one precursor of CO. Recent advances also indicate how the machineries responsible for cofactor generation obtain iron. Several transport systems for iron into bacterial cells exist; however, in *Escherichia coli*, it is mainly the ferrous iron transporter Feo and the ferric-citrate siderphore system Fec that are involved in delivering the metal for Hyd biosynthesis. Genetic analyses have provided evidence for the existence of key checkpoints during cofactor biosynthesis and enzyme assembly that ensure correct spatiotemporal maturation of these modular oxidoreductases.

Keywords: carbon monoxide; cyanide; diatomic ligands; Isc (iron sulfur cluster) machinery; [NiFe]-hydrogenase maturation.

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Introduction

With a redox potential of around -420 mV for the $H_2/2H^+$ couple, hydrogen is an ideal electron donor to drive redox processes in biological systems. Equally, proton reduction to hydrogen can be used as a means of removing excess reducing equivalents from biological systems generated through oxidation of reduced carbon substrates. In contrast to protons, hydrogen diffuses readily across biological membranes, and this forms a simple means of generating a proton gradient (1). These properties of hydrogen have meant that this molecule has been, and remains to this day, a major driver for sustaining the redox processes that govern life on earth.

The term hydrogenase (Hyd; EC 1.12.1.2) was introduced by Stephenson and Stickland (2) to describe the enzyme that catalyses the reversible oxidation of molecular hydrogen to protons and electrons. Hydrogenases are ubiquitous and found in all three domains of life (3, 4). Phylogenetic analysis of the primary structure of the catalytic subunit revealed that Hyd enzymes have evolved as three independent classes (5) further emphasizing the significance of hydrogen metabolism for the evolution of life on our planet. These three classes use different combinations of metal ion cofactors to activate hydrogen and have been named based on the metals found in their respective catalytic center. They include the [NiFe]-Hyd found in bacteria and archaea, [FeFe]-Hyd found in eukarya and bacteria and [Fe]-Hyd, so far found only in certain methanogenic archaea (3, 4). All of these enzymes have in common a strict requirement for iron, and this metal is essential for catalysis. In the [NiFe]-Hyd and [FeFe]-Hyd classes, iron is also necessary for electron transfer processes within these enzymes.

Frequently, [NiFe]-Hyd are modular heteromeric enzymes comprising a large subunit, where catalysis occurs, and a small subunit, which relays electrons to and from the active site (Figure 1). The bimetallic active site is buried deeply within the large subunit to which molecular hydrogen diffuses through specific gas channels (6–8). This metal center catalyzes the endergonic (200 kJ mol^{-1}

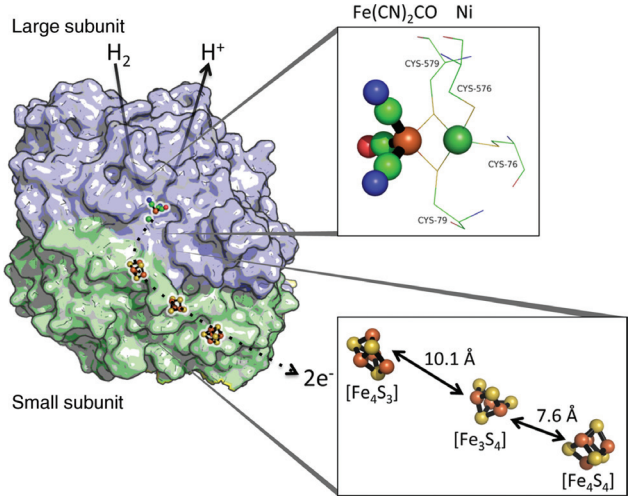

Figure 1 Structure of [NiFe]-hydrogenase 1 from *E. coli*.
The figure presents a heterodimer of Hyd-1, which is actually a dimer of dimers (23), to highlight the overall structure and organization of the cofactors within the enzyme's core. The large subunit harbors a Ni-Fe(CN)$_2$CO site that is coordinated by four Cys residues, and the small subunit contains various types of Fe-S clusters. A large interface between the large and small subunits ensures proximity of the active site with the proximal Fe-S cluster. Structural depictions are based on PDB structure 3UQY from *E. coli* Hyd-1 (23).

at 20°C), heterolytic bond cleavage into a proton and a hydride ion (9). The two electrons derived from proton release are channeled through the small subunit via a series of electron carriers, which are usually iron-sulfur (Fe-S) clusters (Figure 1), to specific physiological electron acceptors (4, 6). Occasionally, the Hyd is linked to a diaphorase module to facilitate hydrogen-coupled NAD^+ reduction (10), or the Hyd can be membrane-associated through a specialized membrane anchor module bearing either Fe-S clusters or cytochromes *b*, which relays the electrons to and from the quinone pool (11).

The first structural analysis of a [NiFe]-Hyd (12) revealed the presence of diatomic ligands associated with the Fe ion in the active site. This finding immediately drew attention to an infrared study (13) in which evidence was presented for the association of the diatomic ligands CO and CN^- with [NiFe]-Hyd. Meanwhile, it has been clearly demonstrated that all [NiFe]-Hyd enzymes characterized, to date, have one CO and two CN^- ligands associated with the Fe ion (14, 15). These diatomic ligands maintain the Fe in a low-spin and low-redox state to facilitate hydrogen activation at the nickel ion. Remarkably, both CO and CN^- are also key ligands to the Fe ions in the active site of [FeFe]-Hyd, while [Fe]-Hyd also has CO liganded to the Fe ion but has a pyridinol group located in the vicinity of the iron, instead of the cyanide ligand (4). These enzymes thus represent a remarkably lucid example of convergent evolution.

From the foregoing, it is immediately apparent that iron is an essential component of all Hyd enzymes. While key aspects of nickel uptake and delivery for active site biosynthesis of the [NiFe]-Hyd enzymes is comparatively well understood [for reviews see (15–19)], considerably less is known regarding the biochemical routes taken by iron and how its specific incorporation into the metal centers of these enzymes occurs. Therefore, this review will focus on our current knowledge regarding biosynthesis of the metal centers in [NiFe]-Hyd with a particular emphasis on what has recently been revealed about the incorporation of iron into the hydrogenases of *Escherichia coli*.

Organization and structure of the metal centers in [NiFe]-hydrogenase

Despite its location deep within the large subunit, the bimetallic active site of [NiFe]-Hyd is within 12 Å of the proximal Fe-S cluster in the small subunit (Figure 1), thus allowing facile electron transfer. Usually, the medial and distal Fe-S cluster named with respect to their positions relative to the active site, are also located at appropriate distances to one another to enable efficient electron transfer between them (Figure 1). With a size in the range of 60–65 kDa, and due to the comparatively large contact surface between the subunits (7, 12), the large subunit has no need for its own Fe-S cluster to mediate electron transfer to the small subunit.

The composition and organization of the Fe-S clusters in the hydrogenase small subunits vary, often depending on the environmental habitat of the microbe that synthesizes the hydrogenase, as well as whether the hydrogenase is involved in energy conservation NAD^+ reduction, or in dissipation of redox equivalents through hydrogen evolution. While the oxygen-sensitive, soluble, periplasmic hydrogen-oxidizing hydrogenases of *Desulfovibrio* species have proximal and distal [4Fe-4S] clusters with a high-potential medial [3Fe-4S] cluster (20), the more oxygen-tolerant, membrane-associated hydrogen-oxidizing hydrogenases of the Knallgas bacterium *Ralstonia eutropha* (also known as *Cupriavidus necator*), and the obligate autotrophic γ proteobacterium *Hydrogenovibrio marinus*, have recently been shown to have a novel, proximal [4Fe-3S] cluster along with a medial [3Fe-4S] cluster and a distal [4Fe-4S] cluster (21, 22). The oxygen-tolerant,

membrane-associated hydrogen-oxidizing Hyd-1 of the facultative anaerobe *E. coli* and Hyd-5 of *Salmonella enterica* also has this complement of Fe-S clusters in its small subunit (Figure 1) (23–25). Coordination of the [4Fe-3S] cluster is achieved by two extra Cys residues that are not necessary for activity but for conferring oxygen tolerance (26, 27). There is convincing evidence that the novel [4Fe-3S] cluster has a function in 'protecting' the [NiFe] active site from oxygen attack by avoiding the slowly reacting and 'unready' Ni-A state and instead adopting only the rapidly activating and 'ready' Ni-B state, in which the Fe-S clusters are electron-rich and thus better able to resist an oxygen attack on the active site (23, 26, 27). Indeed, recent evidence also suggests that the [4Fe-3S] cluster functions together with the high-potential medial [3Fe-4S] to facilitate the direct two-electron reduction of oxygen to water (24).

[NiFe]-Hyd enzymes involved in hydrogen evolution, such as Hyd-3 of *E. coli*, have a small subunit predicted to carry a single [4Fe-4S] cluster (Figure 2) (28, 29). Similarly, the HoxY small subunit of the bidirectional Hyd of cyanobacteria is predicted to have a single [4Fe-4S] cluster (30).

Ni-Fe(CN)$_2$CO biosynthesis and maturation of the large subunit

The ability to purify a soluble HycE subunit in the absence of further proteins of the associated formate hydrogenlyase complex (FHL) (Figure 2) led to the establishment of a maturation model of [NiFe]-Hyd by Böck and colleagues (15, 18). The term maturation encompasses both the biosynthesis and the insertion of the [NiFe] site, and this requires a specific set of accessory maturation proteins, which have been termed Hyp (hydrogenase pleiotropic) because a mutation in any one of the key *hyp* genes results in a strain devoid of active [NiFe]-Hyd (15, 18). The core Hyp proteins are HypC, HypD, HypE, and HypF, and together, these proteins synthesize the Fe(CN)$_2$CO center (Figure 3) (15, 31). The HypA and HypB proteins are required for delivery and insertion of the Ni^{2+} ion subsequent to the insertion of the completed Fe(CN)$_2$CO moiety into the active site of the large subunit. Frequently, paralogs of certain Hyp proteins are found, particularly if the microorganism in question synthesizes multiple [NiFe]-Hyd. Thus, for example, mutations in *hypC* and *hypA*

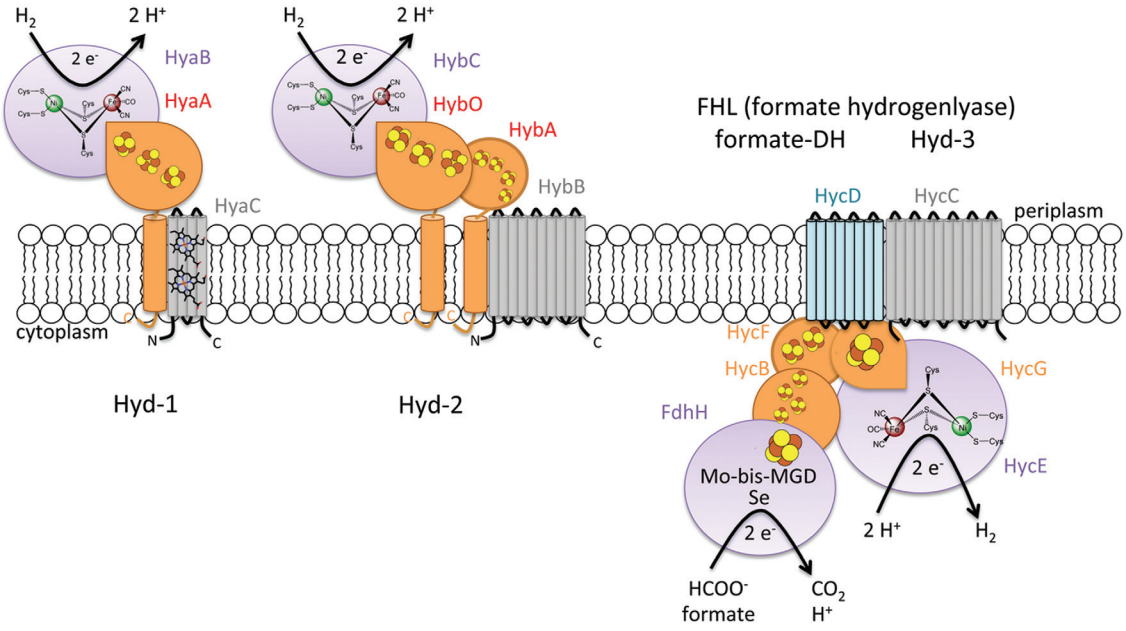

Figure 2 Arrangement of [NiFe]-hydrogenases in the *E. coli* cytoplasmic membrane [modified from ref. (18)].
The H_2-oxidizing enzymes (Hyd-1 and Hyd-2) have their active sites located within the periplasm, while Hyd-3 is part of the formate hydrogenlyase (FHL) complex that is oriented toward the cytoplasm and catalyzes H_2 production. The large subunit harbors a catalytic [NiFe]-site and is shown in purple. The formate dehydrogenase H (Fdh-H) subunit contains a selenocysteine (Se) and a molybdopterin guanine dinucleotide (Mo-bis-MGD) in its active site. The respective small subunits and further electron-transferring subunits are shown in orange, and membrane subunits are shown in gray or blue. The Fe-S clusters are shown as brown (Fe) and yellow (S) circles, while heme *b* molecules are shown as stick models.

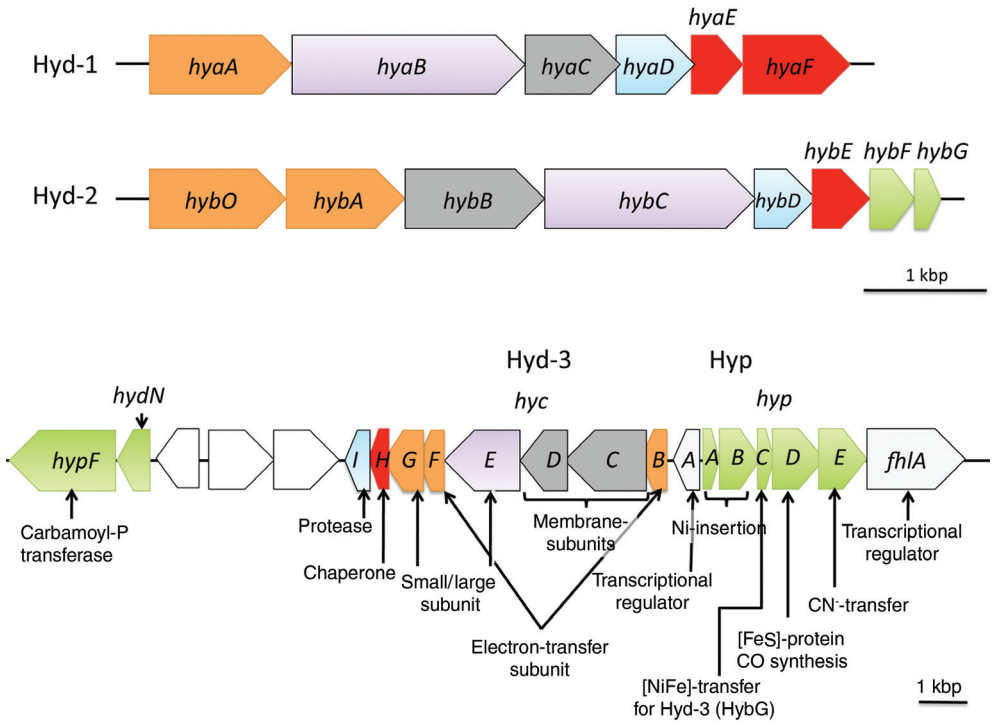

Figure 3 The structural and accessory hydrogenase genes of *E. coli*.
The *hya* (Hyd-1) and *hyb* (Hyd-2) operons along with the genomic region encompassing the *hyc* (Hyd-3) and *hyp* (accessory maturation enzymes) operons are shown. Use of the same color signifies similar properties or function of the respective gene product and are orange for Fe-S-proteins, purple for catalytic subunits, gray for membrane subunits, blue for hydrogenase-specific proteases, red for chaperone proteins, and green for general and hydrogenase-specific accessory proteins that catalyze the biosynthesis and insertion of the [NiFe]-active site. The gene *fdhF*, which encodes the Fdh-H component of the FHL complex, is encoded on a different part of the chromosome (29). The transcriptional regulators *fhlA*, encoding FhlA (formate hydrogenlyase activator), and *hycA* are shown in light gray, and these proteins control expression of the divergent *hyc* and *hyp* operons and the *fdhF* gene (29, 137). The biochemical function of the Fe-S protein encoded by *hydN* is unknown.

prevent maturation of Hyd-3 in *E. coli* but not the maturation of Hyd-1 or Hyd-2. This is because proteins homologous to HypC and HypA, which are required for Hyd-1 and Hyd-2 maturation, are encoded by the *hybG* and *hybF* genes, respectively (Figure 3) (32, 33).

Origin and assembly of the diatomic ligands on the Fe atom

Pioneering studies by the group of Böck demonstrated that the accessory proteins HypC, D, E, and F form a complex, which ultimately assembles the iron atom with its diatomic ligands, and this is inserted into HycE, the catalytic subunit of Hyd-3 (34). It is assumed that this mechanism is universal, with perhaps slight modifications, for all microbes synthesizing [NiFe]-Hyd.

One of the substrates required for diatomic ligand formation is carbamoyl phosphate, which is derived metabolically from citrulline and which has been shown by labeling experiments with ^{13}C-citrulline to be exclusively the source of the two CN^- ligands (35, 36). The first step in the generation of the CN^- ligand involves the activation of carbamoyl phosphate by HypF, which adenylates and therefore stabilizes the carbamoyl group (37). Using the energy derived from hydrolysis of a further ATP HypF transfers the carbamoyl moiety from carbamoyladenylate onto the *C*-terminal cysteine (Cys-322) of HypE generating a thiocarboxamide (38–40). The HypE accessory protein then catalyzes the ATP-dependent dehydration of the thiocarboxamide to a thiocyanate, which is thus activated for transfer onto an iron atom (37–40). Through the use of a *carAB* mutant (devoid of carbamoyl phosphate synthetase), it was clearly demonstrated that neither the CN^- nor the CO ligands were transferred to the HypCD scaffold complex (see also below), despite only CN^- ligand synthesis being impaired (41). This suggests CN^- ligand biosynthesis precedes the synthesis and incorporation of the CO ligand, although it still cannot be ruled

out that the addition of the CO and CN^- moieties could be codependent.

While *E. coli* cells grown in the presence of exogenously supplied ^{13}CO gas inserted the ligand quantitatively into the active site of Hyd-2 or heterologously synthesized regulatory hydrogenase from *R. eutropha* (35, 36, 42), it could be demonstrated unequivocally that the concentration of the gas in the atmosphere was insufficient for it to be the direct source of the ligand (35, 36, 42). Therefore, the precursor of the CO ligand must have a metabolic origin. For heterotrophically growing *R. eutropha*, labeled 1,3-$^{13}C_2$-glycerol was shown to serve as an indirect source for the CO ligand to the regulatory hydrogenase; however, with the multitude of metabolic pathways glycerol can enter, it remains unclear what the immediate precursor is (42).

The iron atom for preassembly of the $FeCN_2CO$ moiety is bound to HypD, but its [4Fe-4S] cluster can be ruled out as a donor (43, 44). Instead, the crystal structure of a HypC and HypD complex (45) suggests that Cys-2 of HypC and Cys-41 of HypD (Cys-38_{HypD} in the structure from *Thermococcus kodakaraensis*) coordinate an Fe ion; however, no iron was observed in the structure presumably because it was labile in the presence of oxygen. Indeed, recent studies have established that strictly anaerobic conditions are necessary to isolate the HypCD complex carrying the $Fe(CN)_2CO$ moiety (45). Notably, this anaerobic complex carries up to two additional iron ions, apart from those in the [4Fe-4S] cluster, suggesting that one of these additional iron ions coordinates the diatomic ligands (46).

Binding studies performed in the presence of oxygen confirmed that a complex between HypC and HypD was able to bind iron between the highly conserved Cys-2 of HypC and Cys-41 (Cys-38 in *T. kodakaraensis*) of HypD, while neither HypC nor HypD alone could interact with the metal (45). More recently, anaerobically isolated *E. coli* HypC and HybG proteins were shown to contain substoichiometric amounts of iron (47). Isolation of either protein aerobically revealed no iron association, confirming the oxygen-labile nature of the bound iron moiety. Moreover, amino acid variants lacking Cys-2 also failed to bind iron, even when the protein was isolated anaerobically confirming that Cys-2 is essential for coordination of the iron ion (47).

Infrared spectroscopy enables visualization of CN^-- and CO-stretching frequencies when these ligands are bound to the Fe atom in the active site (13), and these signals can also be detected on a HypCD complex (see model in Figure 4) (31, 41). A closer examination of the IR spectrum of the anaerobically isolated HypCD complex revealed an additional signature, which could be correlated with the stretching frequency associated with iron-bound CO_2 (46). This observation immediately suggested CO_2 as the possible direct precursor of CO, possibly being reduced by electrons channeled through the Fe-S cluster of HypD. Moreover, although HypD in the absence of HypC can bind the $Fe(CN)_2CO$ cofactor stably, no evidence for bound CO_2 was obtained via IR spectroscopy, and only maximally, five iron ions could be identified associated with the protein (31), suggesting perhaps that a key role for HypC might be the delivery of both the iron and CO_2 to the HypCD complex. Recent studies, indeed, confirmed that anaerobically isolated HypC and HybG proteins carry CO_2 bound to iron (47). Further experiments are, however, required to demonstrate that both the iron and the CO_2 bound by HypC are the direct precursors of the corresponding Fe ion and CO ligand in the $Fe(CN)_2CO$ moiety. Moreover, exogenously supplied ^{13}C-hydrogen carbonate could not be incorporated as a CO ligand to hydrogenase by *E. coli* Δ*carAB* cells (36), while ^{13}C-acetate (labeled at the α-carbon) resulted in labeled CO ligands in *Allochromatium vinosum* (48). This would be commensurate with the earlier proposal that the source of the CO ligand might be endogenously produced CO_2 (48). Thus, a HypC interaction partner that delivers CO_2 needs to be identified.

Intermediate scaffold complexes in the biosynthesis of oxygen-tolerant hydrogenases

Early studies on the hydrogenase system in the endosymbiotic bacterium *Rhizobium leguminosarum* led to the identification of HupK, which shares common structural motifs with the large subunit of [NiFe]-hydrogenases (49). While not a hydrogenase *per se*, HupK was insightfully proposed to have a scaffold function during maturation. More recent studies in a number of bacteria, including *R. eutropha* (50) and *Thiocapsa roseopersicina* (51), which synthesize aerotolerant hydrogenases, have indeed confirmed a scaffold function for HupK. Moreover, these bacteria typically also synthesize at least one additional HypC paralog, which interacts with the HupK homolog and with the precursor form of the large subunit. In *R. leguminosarum*, this HypC homolog, termed HupF (52), carries a *C*-terminal extension, which confers upon the protein the ability to protect the hydrogenase large subunit from the deleterious effects of oxygen during maturation. A model based on work in *R. eutropha* was proposed, in which a complex comprising HoxV (homolog of HupK) and HoxL (homolog of HupF) accept the oxygen-labile $Fe(CN)_2CO$

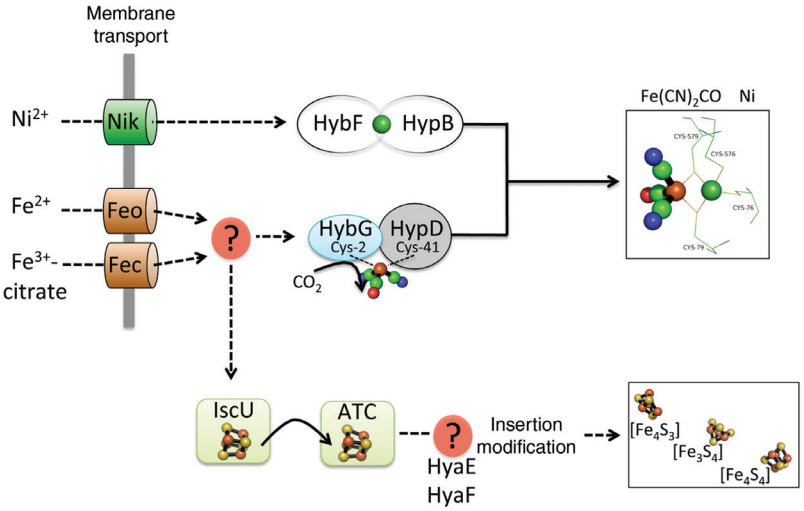

Figure 4 Routes of nickel and iron delivery to the respective large and small subunits.
The nickel and iron ions are selectively transported across the membrane by the NikABC transporter, and either the Feo or Fec transporters, respectively. Free Fe^{2+} is not present in the cytoplasm, and presumably, it is therefore picked up from the respective transporter and delivered to the target proteins by, so far, unknown factors (indicated by a question mark). In the case of the active site iron ion, this might be sequestered by the HypC paralog in *E. coli* HybG, which is required for Hyd-1 large subunit maturation. The $Fe(CN)_2CO$ moiety of the active site is first synthesized on a scaffold comprising HybG (HypC) and HypD. The maturation proteins HypE and HypF supply the cyanide ligands generating them from carbamoyl phosphate and form part of the biosynthetic complex (not shown), while HybG (HypC) and HypD supply the CO ligand, possibly derived from endogenous CO_2. Only after insertion of the $Fe(CN)_2CO$ moiety into the apo-large sunbunit has occurred can HyaB supply the Ni^{2+}, which is inserted by HybF (HypA paralog) and HypB. The small subunit contains various types of Fe-S clusters which are delivered by A-type carrier proteins (ATC) subsequent to their synthesis on the IscU-IscS scaffold system depicted by IscU only. The sulfur is derived from cysteine via the action of the cysteine desulfurase IscS (not shown), but the direct source of the iron ions is not known. Although the proteins directly involved in modification and insertion of the Fe-S clusters have not been identified yet (indicated by a question mark), the accessory proteins HyaE and HyaF as well as binding the Tat signal peptide might have an additional role in this regard.

moiety from the HypCD complex and deliver it to the apo-form of the hydrogenase large subunit (50).

Closure of the active site

The insertion of the $Fe(CN)_2CO$ has been clearly shown to precede the insertion of the nickel ion into the large subunit (53–55). In order to insert the nickel ion into the hydrogenase large subunit, which has been shown most clearly for Hyd-3 in *E. coli*, the proteins HypA, HypB, and SlyD are required, whereby the HypB GTPase dimerizes in a GTP-dependent manner to transfer the nickel ion, while HypA serves as a scaffold protein (Figure 4) (56–58). SlyD is a peptidyl-prolyl *cis*/*trans* isomerase, and although not essential for hydrogenase maturation, it facilitates the release and transfer of the Ni^{2+} ion as well as mediating the interaction of HypB with HycE (59, 60). Because a *hypB* mutant can be phenotypically complemented through the addition of high amounts of nickel to the growth medium (61), this indicates that the HypAB-SlyD nickel-insertion machinery has a kinetic role in nickel insertion. In bacteria such as *Helicobacter pylori*, HypA and HypB are also responsible for delivery and insertion of the nickel ion into the active site of urease. Notably, *H. pylori* mutants lacking the *hypAB* genes have reduced pathogenicity (19, 62).

Completion of hydrogenase large subunit maturation [an exception being the regulatory hydrogenase of *R. eutropha* (63)] requires the proteolytic removal of a *C*-terminal peptide [reviewed in (15)]. The sequence and length of these *C*-terminally cleaved peptides vary between hydrogenases, and each large subunit has its own highly specific endoprotease (15). Proteolytic processing of the large subunit by the endoprotease likely leads to a conformational change that encloses the active site after its delivery by HypC (Figure 5) (64). The sequence of the *C*-terminal peptide is not highly conserved, probably reflecting the requirement for a specific endoprotease for each hydrogenase, but its presence is essential for nickel insertion. Proteolysis does not happen in the absence of nickel, and therefore, the metal is a key determinant that governs

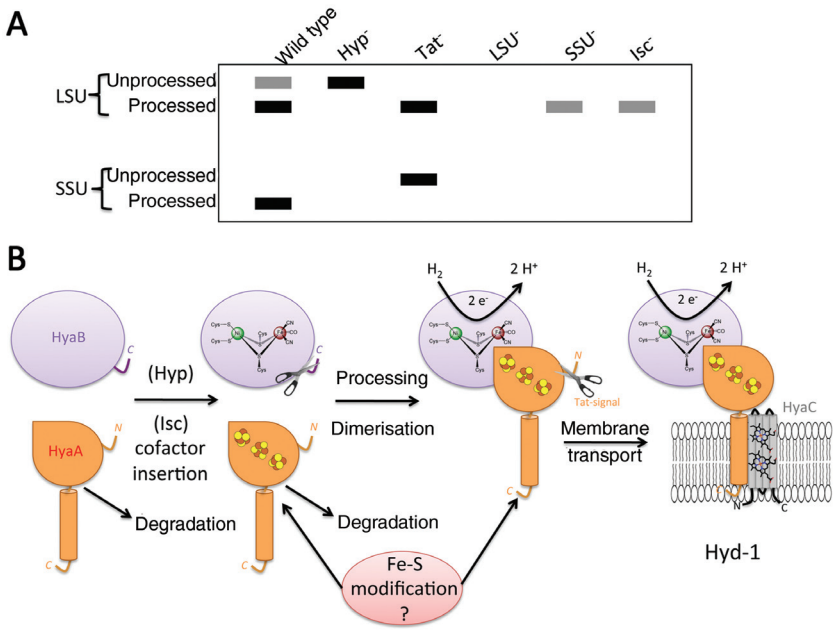

Figure 5 Order of assembly events as depicted exemplarily for *E. coli* hydrogenase 1.
(A) A summary of the observed processing pattern for large (LSU) and small subunit (SSU) of hydrogen-oxidizing [NiFe]-Hyd-1 in *E. coli* in various mutant backgrounds is shown. (1) In the total protein fraction of anaerobically grown wild-type cells, a mixture of mature and unprocessed LSU, together with processed SSU can be observed. (2) Absence of the Hyp protein machinery results in unprocessed LSU due to lack of [NiFe]-cofactor insertion. The SSU is not processed by the Tat machinery, is rapidly degraded, and is usually not visible (72). (3) Processing of the SSU takes place during Tat transport and does not occur in the absence of the Tat system. The LSU is, however, processed and contains its active site. The LSU and SSU interact, and Hyd activity is detectable in the cytoplasm with artificial electron dyes as shown for *R. eutropha* (138). (4) A similar phenotype to that shown by a Hyp$^-$ mutant is observed in the absence of the LSU as binding partner where the SSU remains unprocessed and is rapidly degraded. (5) In the absence of the SSU, the processing of the LSU still occurs and retains stability; however, the protein remains in the cytoplasm (139). (6) Deletion of the A-type carrier components of the Isc system results in a lack of SSU and reduced levels of the processed LSU (72).
(B) The large subunit of Hyd-1, HyaB, and the small subunit, HyaA, are synthesized, the respective cofactors are inserted, the large subunit is processed prior to assembly with the small subunit, and the small subunit is only processed during membrane transport of the heterodimer. Several quality control checkpoints lead to protein degradation if maturation cannot proceed. It is unknown when and how the conversion of the proximal [4Fe-4S]-cluster to a [4Fe-3S] takes place.

protease function (65–69). The specific endoproteases for Hyd-1 and Hyd-2 are HyaD and HybD, respectively (Figure 3) (70). As proteolytic processing only occurs when nickel, and no other divalent cation (68, 71), is present, this indicates that whenever processing is observed, a *bona fide* Ni-Fe(CN)$_2$CO active site is present (Figure 5A). It has been observed that in the absence of this proteolytic processing event, or in the absence of the large subunit, the small subunit is rapidly degraded (72). This indicates a tight coupling between large and small subunit processing.

Fe-S cluster biosynthesis

Iron-sulphur proteins have various functions ranging from regulation of gene expression to structural and electron-transferring roles. The modular design of oxidoreductases shows an array of Fe-S clusters in their small subunits separated by less than ≈14 Å (73), which is shown in Figure 1 for hydrogenases. To ensure efficient electron transfer, some large modular oxidoreductase, such as formate dehydrogenases and nitrate reductases, require an additional Fe-S cluster in their large subunit (73, 74). Assembly and insertion of Fe-S clusters are not spontaneous processes, but there is also not a dedicated protein for every single Fe-S cluster type. Instead, three general and independent machineries for biosynthesis of Fe-S clusters have evolved (75, 76). Studies on nitrogenase biosynthesis in *Azotobacter vinelandii* identified the NifU and NifS proteins, which have a key role in Fe-S and FeMo cofactor biosynthesis (77–79). A further set of proteins referred to as the Isc (iron sulfur cluster) machinery was subsequently discovered to be encoded on the genome of *A. vinelandii* (75, 76). Meanwhile, the Isc system has been discovered in numerous microorganisms, including *E. coli*, together with the orthologous Suf (sulfur assimilation) Isc

biosynthetic machinery (76, 80, 81). The Suf system is comprised of the six gene products of the *sufABCDSE* operon, which is mainly expressed under oxidative stress (82). The *isc* operon, on the other hand, is negatively regulated by its own regulator IscR and comprises the genes *iscRSUA-hscBA-fdx-iscX* (83, 84). Both systems have homologous components. Sulfur is recruited from cysteine by the desulfurases IscS or SufS and then assembled into an unstable [2Fe-2S] cluster on the scaffolds IscU or SufBCD, respectively (75, 85). The physiological iron source, although unknown, can be supplied *in vitro* in the form of free Fe^{2+} or can be delivered on the frataxin-like protein CyaY (Figure 4). The potential role of CyaY *in vivo* has only been suggested so far through the demonstration of an interaction between CyaY and IscS, as well as through its *in vivo* regulatory role (86–89). Transfer of the completed Fe-S cluster to the apo-protein target is probably accomplished by the A-type carrier (ATC) proteins IscA, SufA, and ErpA, whose functions have been studied through a combination of mutagenesis, protein-protein interaction, and *in vitro* Fe-S cluster transfer studies (90). It has been shown that for certain key anaerobic respiratory enzymes, the Suf system plays no significant role, while the Isc system is crucial for the generation of active respiratory hydrogenases (72, 74). Strains devoid of the IscA, ErpA, or IscU proteins lack activity of Hyd-1 and Hyd-2, and this is mainly due to the absence of the small subunits (72). Notably, in *iscA* and *erpA* mutants, the large subunits of these enzymes are nevertheless processed, indicating that the Isc system is not solely responsible for active site iron insertion (Figure 5A) (72).

The activity of the FHL complex was shown to be reduced when the A-type carrier (ATC) proteins were missing, but was absent only when the gene coding for the scaffold protein IscU or when both the *iscA* and *erpA* genes were deleted (91). The latter observation indicates partial redundancy of function between IscA and ErpA. The level of the respective Hyd-1 and Hyd-2 large subunits was significantly reduced in the *isc* mutants (Figure 5A). This suggests either that enhanced degradation of the large subunit in the absence of matured small subunit occurred or possibly that expression of the *hya* and *hyb* operons was reduced due to an incompletely functional Isc system. It has been shown that the IscR regulator represses *hya* and *hyb* operon expression under aerobic conditions (92, 93), and deletion of the *iscR* gene causes derepression of *hya* and *hyb* expression (94). Nevertheless, it has been demonstrated recently (95, 96) that both apo-IscR and [2Fe-2S]-IscR have different regulatory functions, and because the presence of the Fe-S cluster in IscR is determined by the activity of the Isc machinery, any mutation affecting Isc function has a knock-on effect on IscR-regulated genes. Future experiments will be required to determine to what extent mutations in *isc* operon genes regulate expression of operons encoding anaerobic oxidoreductases.

In a recent study, it was revealed that the Isc system is also able to provide basic [4Fe-4S] clusters during the heterologous expression of [FeFe]-Hyd in the absence of their specific maturases (97, 98). Moreover, apparently, the Isc system can be forced to erroneously introduce an oxygen labile Fe-S cluster into the active sites of HyaB and HybC, the large subunits of the [NiFe]-hydrogenases Hyd-1 and Hyd-2, respectively. This cluster is coordinated by the four cysteinyl residues that normally ligand the [NiFe]-cofactor (99) and appears to be introduced when the maturation machinery cannot insert the [NiFe]-cofactor, thus possibly serving to stabilize the protein during maturation. These observations raise the intriguing question as to how the erroneous insertion of Fe-S clusters is prevented, and indeed, what governs delivery and construction of the highly diverse Fe-S clusters like those found in the small subunits?

Subunit assembly and membrane transport: the checkpoints

Generally, H_2-uptake Hyd that couple H_2 oxidation to energy conservation are membrane-associated with their catalytic large subunits located in the periplasm. This necessitates translocation of the mature enzyme across the membrane by the twin-arginine translocation (Tat) machinery (100, 101). In contrast, Hyd enzymes located in the cytoplasm (see Hyd-3 in Figure 2) often catalyze H_2 production, and these do not rely on the Tat machinery for the completion of their assembly (11).

The H_2-oxidizing Hyd-1 of *E. coli* is encoded by the *hyaABCDEF* operon. The first gene, *hyaA*, encodes the small Fe-S cluster containing subunit, while the second gene, *hyaB*, encodes the catalytic subunit, which harbors the [NiFe]-active site (Figure 3) (102). The HyaA, but not the HyaB, polypeptide carries an *N*-terminal Tat signal sequence, and this is responsible for translocation of the HyaA-HyaB heterodimer (or possibly dimer of dimers) across the membrane via a 'piggy-back' mechanism (103). Similarly, the first gene of the *hybOABCDEFG* operon encodes the small subunit of Hyd-2, and HybO also has a Tat signal sequence (Figure 3) (104, 105). The small subunits of both enzymes are also anchored in the membrane via a hydrophobic *C*-terminal α-helix; however, electrons are transferred to the quinone pool via a further membrane-integral subunit, which harbors a *b*-type heme cofactor in the case of Hyd-1 (HyaC). Unusually, in the case

of Hyd-2, the membrane anchor HybB lacks any apparent cofactor (Figure 2) (11, 106). However, Hyd-2, which has an additional ferredoxin-like subunit that has an *N*-terminal Tat signal peptide, is required for electron transfer to other membrane-associated oxidoreductases and appears to be translocated across the membrane independently of the HybO-HybC complex (106).

Owing to their order in the operon, the genes encoding the small subunits of the H_2-oxidizing Hyds are transcribed and translated prior to those of the large subunits. However, if the maturation of the large subunit is not completed, the Tat signal peptide of the small subunit is not removed, the small subunit protein is rapidly degraded, and the unprocessed large subunit remains in the cytoplasm (Figure 5) (72). If the Fe-S cluster of the small subunit cannot be introduced due to a defective Isc machinery, the small subunit is also rapidly degraded, and the large subunit also remains in the cytoplasm; notably, the maturation of the large subunit does not depend on whether maturation of the small subunit occurs or not (72). Thus, the maturation of the large subunit must be completed first and only then is the maturation of the small subunit completed, presumably concomitant with transport through the Tat translocon. It is likely that the interaction of the complex with the Tat system also controls protein turnover of the small subunits, and a system must be in place that 'masks' the Tat signal peptide from the Tat machinery until the complex is ready for transport. Private chaperones in *E. coli* specifically bind the small subunit precursor, and they are encoded by the genes *hyaE*, *hyaF*, and *hybE* in the operons of the respective H_2-oxidizing Hyd (Figure 3). Homologs of HyaE and HyaF, called HupG and HupH, respectively, were shown to be essential for maturation of the small subunit of hydrogenase in *R. leguminosarum* (107), while in *R. eutropha*, the HyaE homolog, HoxO, interacts with the signal peptide of the small subunit HoxK (108).

The structure of HyaE has been determined, and it belongs to a class of proteins with a thioredoxin fold; however, they lack the Cys residues necessary for redox reactions (109). Notably, a *hoxO* mutant of *R. eutropha* fails to synthesize active membrane-bound Hyd, and the mutation caused the precursor of the large subunit (HoxG) to be delivered prematurely to the membrane, while processed HoxG was detectable in the cytoplasm (110). This suggests that the HyaE/HoxO proteins coordinate maturation of the small subunit after large subunit processing has occurred and deliver the completed complex to the Tat machinery.

In *E. coli*, single *hyaE* or *hyaF* deletions have no apparent defects in respiratory hydrogenase assembly and membrane targeting (106); however, when both gene products are absent, no Hyd-1 activity is observed indicating that some redundancy in function between the proteins exists (102). The *R. eutropha* HyaF homolog HoxQ influences large subunit maturation, and it was suggested that it is necessary for other key steps of small subunit maturation (110), while in *R. leguminosarum*, the HupH protein (homolog of HyaF in *E. coli*) was shown to interact specifically with the premature small subunit (107). Owing to the fact that the hydrogenases of *R. eutropha* are synthesized under aerobic conditions, it is likely that the bacterium has further chaperones, e.g., HoxR and HoxT, which protect the Fe-S cluster in the small subunit from oxygen damage. Indeed, it was shown that the rubredoxin HoxR plays a role in establishing the final complement of Fe-S clusters in the small subunit (111, 112). Such a function is unnecessary in *E. coli* because the hydrogenases are only synthesized under anaerobic conditions, and this explains why no homologs of HoxR and HoxT exist in *E. coli*.

The HybE protein was shown to recognize specifically the Tat signal sequence of the small subunit HybO as well as the *C*-terminal extension of the large subunit HybC (113). The protein is not required for maturation of the large subunit HybC but for coordinated transport of both subunits to the membrane, as in Δ*hybE* strains, the small subunit was found in the membrane without the catalytic partner HybC (103).

A dual role for these accessory proteins in the modification of the proximal [4Fe-3S] cluster has been suggested (25) as well as in protection against reactive oxygen species (112) (Figure 4). Possibly, these hydrogenase-specific accessory proteins, together with the local amino acid sequence of the small subunit protein, determine the type of cluster that is inserted. Using model peptides mimicking the local amino acid structure of an Fe-S protein, it has been shown that amino acid exchanges influence the occurrence of particular Fe-S cluster types (114).

In contrast to these findings, the cytoplasmically oriented FHL complex is more tolerant toward defects in Fe-S cluster biosynthesis (91). Unfortunately, little is known about the assembly of the FHL complex and the stoichiometry of its components, except that none of the subunits harbors a signal sequence for membrane transport. It makes sense that only correctly inserted Fe-S cluster-containing proteins are allowed to pass through the Tat machinery, while FHL proteins can partially assemble prior to processing (C.P. and F. Sargent, unpublished data).

Uptake of metals and regulation

The uptake of metal ions from the medium is highly specific in microorganisms and independent of the

concentration of other metals in the medium (115). The most abundant metals of the 343 cytoplasmic metal proteins in *Pyrococcus furiosus* are iron and zinc (97%) followed by tungsten and nickel (<2.5%) with <0.5% of other metals (115). Transport and insertion of the nickel ion into the active site of Hyd has been well investigated. A specific nickel ABC (ATP binding cassette) transporter was discovered through mutagenesis studies, and mutations that prevent its synthesis reduce [NiFe]-Hyd activity. Nevertheless, this reduction in hydrogenase activity can be phenotypically complemented through the addition of high amounts of nickel to the growth medium, indicating nonspecific uptake of nickel via other transport systems (17, 116, 117). Expression of the *nik* genes encoding the nickel transporter is Fnr dependent and is repressed by the nickel-responsive transcription factor NikR in the presence of high intracellular nickel (17, 117, 118). Fnr has an oxygen-labile Fe-S cluster, and it ensures that, in *E. coli*, the *nik* operon is only expressed anaerobically when the hydrogenases are synthesized (Figure 4).

While the Nik ABC transporter is the only transporter specific for nickel uptake in *E. coli*, an additional nickel permease is present in *H. pylori* (119). *R. leguminosarum* also encodes a nickel permease, HupE (120), while a homolog, HoxN, transports nickel by a uniport mechanism in *R. eutropha* (121). Intriguingly, *Salmonella enterica* has an aerobically synthesized Hyd enzyme, which contains a fully functional [NiFe]-active site ((122); C.P. and F. Sargent, unpublished results), suggesting that either Ni^{2+} uptake occurs aerobically, or sufficient stores of the ion are available to satisfy aerobic enzyme biosynthesis.

The mechanisms governing iron uptake are much more diverse in *E. coli*. There are high-affinity uptake systems for ferrous iron (FeoABC), various siderophore-mediated uptake systems (FecA-E, FepA-G, FhuA-D), and further uptake systems that transport iron nonspecifically (123–125). Iron homeostasis is controlled by the iron-responsive transcription factor Fur (ferric uptake regulator) (126). Fur binds iron, thus, enabling it to bind to a consensus sequence in the -35 region of promoters of iron-responsive genes repressing transcription (127). Surprisingly, a *fur* deletion strain of *E. coli* shows reduced FHL activity due to lower transcription levels of the *hyc* genes, while transcription of the genes coding for the H_2-uptake Hyd remains unaffected (128). An inverse effect is observed when iron uptake cannot be accomplished. Strains defective in the Feo system have drastically reduced Hyd-1 and Hyd-2 levels with only small amounts of large subunit detectable, while Hyd-3, and thus FHL, levels are less significantly affected (Figure 4) (129). Only further deletion of genes coding for the iron citrate transporter, Fec, and genes of the enterochelin biosynthesis pathway cause a reduction in FHL activity, revealing a distinct bias for maintaining different Hyd activities within the cell under specific conditions (129).

Expert summary

[NiFe]-hydrogenases are evolutionarily ancient enzymes. Examination of their active sites reveals that all components derive from inorganic compounds abundant on early earth (130) including nickel and iron sulfides, carbon monoxide, and cyanide. Conceivably, somehow these components formed an early primitive catalyst that was able to generate hydrogen in the reducing and acidic environment or to activate volcanic hydrogen gas; evolution has retained and adapted these compounds as key catalytic components of modern-day hydrogenase. Iron remains the key component, and the evolution of a specific set of accessory proteins has facilitated the biosynthesis of these iron-based cofactors from metabolic intermediates as well as their assembly into the appropriate apo-protein 'cage', which considerably enhances their catalytic efficiency and assures stability by protecting them from the bulk phase. One future challenge will be to design equally efficient catalysts that are considerably more robust toward aqueous environments.

Recent collaborative efforts between chemists, biochemists, spectroscopists, and biologists (24, 131, 132) have highlighted the rapid advances that can be achieved in understanding the chemistry and biology of hydrogen activation by combining expertise. The challenge for the future will be to develop these synergies further to provide a complete understanding of [NiFe]-hydrogenase biosynthesis. Much new and surprising biochemistry awaits discovery in this important scientific area.

Outlook

What is becoming increasingly apparent is that biosynthesis of cofactors takes place on assembly platforms or scaffolds often comprising numerous accessory proteins (31, 41, 89, 133). Each of these accessory proteins probably has one or more specific biochemical function, but each is also important for the integrity and functionality of the complex. Moreover, there are components of this platform that have the job of specifically recognizing the appropriate apo-protein target for cofactor delivery. The private chaperones of apo-protein targets presumably have the role of 'masking' peptide sequences, e.g., binding the Tat signal peptide to prevent premature translocation of an 'immature' protein through the Tat machine. But these chaperones might also

act as guides, or docking sites, for cofactor delivery proteins, ensuring the correct cofactor is delivered to the correct site at the appropriate time. Considering the fact that the cytoplasmic protein concentration is likely to be ≥250 mg/ml (134), it is probable that the maturation of complex metalloenzymes such as [NiFe]-hydrogenases occurs on large multiprotein complexes that include all necessary components. These complexes are likely to be highly localized, possibly membrane-associated, particularly for those that are translocated across the cytoplasmic membrane by the Tat translocon. Further development of high-resolution cell biological methods will facilitate elucidation of these complexes and their cellular localization. The development of X-ray free-electron laser technology (135), single-molecule studies together with methods such as high-resolution secondary ion mass spectrometry (nano-SIMS) (136) will resolve the structures of these complexes, the physical interactions between components, and the individual biochemical steps on these biosynthetic pathways.

With knowledge gained from the structural and spectroscopic analyses of hydrogenases, it will be possible to make major advances in constructing robust and highly efficient hydrogenase biomimics (131, 132), greatly facilitating the development of hydrogen-driven fuel cell technology as one of several future alternative clean energy sources.

Highlights

- [NiFe]-hydrogenases harbor Fe in different types of Fe-S clusters and in the active site, which also has a nickel ion.
- The Fe-S clusters are inserted by the Isc system; however, the mechanism underlying the insertion and modification of the clusters is unknown.
- Active site iron is decorated with CO and CN^- diatomic ligands on a maturation protein scaffold complex comprising HypC, HypD, HypE, and HypF.
- Iron and CO_2 are bound by the HypC protein and might represent intermediates in active site biosynthesis.
- The Feo transport system is the main delivery route of iron for [NiFe]-hydrogenase cofactor biosynthesis in *E. coli*.
- Iron metabolism not only influences hydrogenase activity by determining cofactor availability but also affects transcription of the hydrogenase structural operons.
- In *E. coli*, assembly of the H_2 uptake hydrogenases is more strictly controlled than that of the cytoplasmic hydrogen-evolving FHL complex, possibly reflecting differential iron requirements, allowing metabolic demands to be met.
- There are several key assembly checkpoints during hydrogenase biosynthesis, which, if not correctly passed, result in subunit turnover.

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References

1. Branscomb E, Russell MJ. Turnstiles and bifurcators: the disequilibrium converting engines that put metabolism on the road. Biochim Biophys Acta 2013; 1827: 62–78.
2. Stephenson M, Stickland L. Hydrogenase: a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme. Biochem J 1931; 25: 205–14.
3. Vignais PM, Billoud B. Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev 2007; 107: 4206–72.
4. Thauer RK, Kaster A-K, Goenrich M, Schick M, Hiromoto T, Shima S. Hydrogenases from methanogenic archaea, nickel, a novel cofactor, and H_2 storage. Annu Rev Biochem 2010; 79: 507–36.
5. Wu LF, Mandrand-Berthelot MA. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. FEMS Microbiol Rev 1993; 10: 243–69.
6. Cammack R. Redox enzymes. Splitting molecular hydrogen. Nature 1995; 373: 556–7.
7. Fontecilla-Camps JC, Volbeda A, Cavazza C, Nicolet Y. Structure/function relationships of [NiFe]- and [FeFe]-hydrogenases. Chem Rev 2007; 107: 4273–303.
8. Liebgott P-P, Leroux F, Burlat B, Dementin S, Baffert C, Lautier T, Fourmond V, Ceccaldi P, Cavazza C, Meynial-Salles I, Soucaille P, Fontecilla-Camps JC, Guigliarelli B, Bertrand P, Rousset M, Léger C. Relating diffusion along the substrate tunnel and oxygen sensitivity in hydrogenase. Nat Chem Biol 2010; 6: 63–70.

9. Kubas GJ. Molecular hydrogen complexes: coordination of a sigma bond to transition metals. Acc Chem Res 1988; 21: 120–8.
10. Schneider K, Schlegel HG. Purification and properties of soluble hydrogenase from Alcaligenes eutrophus H 16. Biochim Biophys Acta 1976; 452: 66–80.
11. Vignais PM, Billoud B, Meyer J. Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 2001; 25: 455–501.
12. Volbeda A, Charon M, Piras C, Hatchikian E, Frey M, Fontecilla-Camps J. Crystal structure of the nickel-iron hydrogenase from Desulfovibrio gigas. Nature 1995; 373: 580–7.
13. Bagley KA, Duin EC, Roseboom W, Albracht SP, Woodruff WH. Infrared-detectable groups sense changes in charge density on the nickel center in hydrogenase from Chromatium vinosum. Biochemistry 1995; 34: 5527–35.
14. Volbeda A, Garcin E, Piras C, De Lacey AL, Fernandez VM, Hatchikian EC, Frey M, Fontecilla-Camps JC. Structure of the [NiFe] hydrogenase active site: evidence for biologically uncommon Fe ligands. J Am Chem Soc 1996; 118: 12989–96.
15. Böck A, King P, Blokesch M, Posewitz M. Maturation of hydrogenases. Adv Microb Physiol 2006; 51: 1–71.
16. Eitinger T, Suhr J, Moore L, Smith JAC. Secondary transporters for nickel and cobalt ions: theme and variations. Biometals 2005; 18: 399–405.
17. Wu L, Navarro C, Mandrand-Berthelot MA. The hydC region contains a multi-cistronic operon (nik) involved in nickel transport in Escherichia coli. Gene 1991; 107: 37–42.
18. Forzi L, Sawers RG. Maturation of [NiFe]-hydrogenases in Escherichia coli. Biometals 2007; 20: 565–78.
19. Kaluarachchi H, Chan Chung KC, Zamble DB. Microbial nickel proteins. Nat Prod Rep 2010; 27: 681–94.
20. Rousset M, Montet Y, Guigliarelli B, Forget N, Asso M, Bertrand P, Fontecilla-Camps JC, Hatchikian EC. [3Fe-4S] to [4Fe-4S] cluster conversion in Desulfovibrio fructosovorans [NiFe] hydrogenase by site-directed mutagenesis. Proc Natl Acad Sci USA 1998; 95: 11625–30.
21. Fritsch J, Scheerer P, Frielingsdorf S, Kroschinsky S, Friedrich B, Lenz O, Spahn CMT. The crystal structure of an oxygen-tolerant hydrogenase uncovers a novel iron-sulphur centre. Nature 2011; 479: 249–52.
22. Shomura Y, Yoon K-S, Nishihara H, Higuchi Y. Structural basis for a [4Fe-3S] cluster in the oxygen-tolerant membrane-bound [NiFe]-hydrogenase. Nature 2011; 479: 253–6.
23. Volbeda A, Amara P, Darnault C, Mouesca J-M, Parkin A, Roessler MM, Armstrong FA, Fontecilla-Camps JC. X-ray crystallographic and computational studies of the O_2-tolerant [NiFe]-hydrogenase 1 from Escherichia coli. Proc Natl Acad Sci USA 2012; 109: 5305–10.
24. Evans RM, Parkin A, Roessler MM, Murphy BJ, Adamson H, Lukey MJ, Sargent F, Volbeda A, Fontecilla-Camps JC, Armstrong FA. Principles of sustained enzymatic hydrogen oxidation in the presence of oxygen – the crucial influence of high potential Fe-S clusters in the electron relay of [NiFe]-hydrogenases. J Am Chem Soc 2013; 135: 2694–707.
25. Parkin A, Sargent F. The hows and whys of aerobic H_2 metabolism. Curr Opin Chem Biol 2012; 16: 26–34.
26. Goris T, Wait AF, Saggu M, Fritsch J, Heidary N, Stein M, Zebger I, Lendzian F, Armstrong FA, Friedrich B, Lenz O. A unique iron-sulfur cluster is crucial for oxygen tolerance of a [NiFe]-hydrogenase. Nat Chem Biol 2011; 7: 310–8.
27. Lukey MJ, Roessler MM, Parkin A, Evans RM, Davies RA, Lenz O, Friedrich B, Sargent F, Armstrong FA. Oxygen-tolerant [NiFe]-hydrogenases: the individual and collective importance of supernumerary cysteines at the proximal Fe-S cluster. J Am Chem Soc 2011; 133: 16881–92.
28. Böhm R, Sauter M, Böck A. Nucleotide sequence and expression of an operon in Escherichia coli coding for formate hydrogenlyase components. Mol Microbiol 1990; 4: 231–43.
29. Sauter M, Böhm R, Böck A. Mutational analysis of the operon (hyc) determining hydrogenase 3 formation in Escherichia coli. Mol Microbiol 1992; 6: 1523–32.
30. Horch M, Lauterbach L, Lenz O, Hildebrandt P, Zebger I. NAD(H)-coupled hydrogen cycling – structure-function relationships of bidirectional [NiFe] hydrogenases. FEBS Lett 2012; 586: 545–56.
31. Stripp ST, Soboh B, Lindenstrauß U, Braussemann M, Herzberg M, Nies DH, Sawers RG, Heberle J. HypD is the scaffold protein for Fe-$(CN)_2$CO cofactor assembly in [NiFe]-hydrogenase maturation. Biochemistry 2013; 52: 3289–96.
32. Hube M, Blokesch M, Böck A. Network of hydrogenase maturation in Escherichia coli: role of accessory proteins HypA and HybF. J Bacteriol 2002; 184: 3879–85.
33. Jacobi A, Rossmann R, Böck A. The hyp operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in Escherichia coli. Arch Microbiol 1992; 158: 444–51.
34. Blokesch M, Albracht SPJ, Matzanke BF, Drapal NM, Jacobi A, Böck A. The complex between hydrogenase-maturation proteins HypC and HypD is an intermediate in the supply of cyanide to the active site iron of [NiFe]-hydrogenases. J Mol Biol 2004; 344: 155–67.
35. Forzi L, Hellwig P, Thauer RK, Sawers RG. The CO and CN^- ligands to the active site Fe in [NiFe]-hydrogenase of Escherichia coli have different metabolic origins. FEBS Lett 2007; 581: 3317–21.
36. Lenz O, Zebger I, Hamann J, Hildebrandt P, Friedrich B. Carbamoylphosphate serves as the source of CN(-), but not of the intrinsic CO in the active site of the regulatory [NiFe]-hydrogenase from Ralstonia eutropha. FEBS Lett 2007; 581: 3322–6.
37. Tominaga T, Watanabe S, Matsumi R, Atomi H, Imanaka T, Miki K. Crystal structures of the carbamoylated and cyanated forms of HypE for [NiFe] hydrogenase maturation. Proc Natl Acad Sci USA 2013; 110: 20485–90.
38. Blokesch M, Paschos A, Bauer A, Reissmann S, Drapal N, Böck A. Analysis of the transcarbamoylation-dehydration reaction catalyzed by the hydrogenase maturation proteins HypF and HypE. Eur J Biochem 2004; 271: 3428–36.
39. Paschos A, Bauer A, Zimmermann A, Zehelein E, Böck A. HypF, a carbamoyl phosphate-converting enzyme involved in [NiFe] hydrogenase maturation. J Biol Chem 2002; 277: 49945–51.
40. Reissmann S, Hochleitner E, Wang H, Paschos A, Lottspeich F, Glass RS, Böck A. Taming of a poison: biosynthesis of the NiFe-hydrogenase cyanide ligands. Science 2003; 299: 1067–70.
41. Bürstel I, Siebert E, Winter G, Hummel P, Zebger I, Friedrich B, Lenz O. A universal scaffold for synthesis of the Fe$(CN)_2$(CO) moiety of [NiFe]-hydrogenase. J Biol Chem 2012; 287: 38845–53.
42. Bürstel I, Hummel P, Siebert E, Wisitruangsakul N, Zebger I, Friedrich B, Lenz O. Probing the origin of the metabolic precursor of the CO ligand in the catalytic center of [NiFe]-hydrogenase. J Biol Chem 2011; 286: 44937–44.

43. Blokesch M, Böck A. Properties of the [NiFe]-hydrogenase maturation protein HypD. FEBS Lett 2006; 580: 4065–8.
44. Watanabe S, Matsumi R, Arai T, Atomi H, Imanaka T, Miki K. Crystal structures of [NiFe] hydrogenase maturation proteins HypC, HypD, and HypE: insights into cyanation reaction by thiol redox signaling. Mol Cell 2007; 27: 29–40.
45. Watanabe S, Matsumi R, Atomi H, Imanaka T, Miki K. Crystal structures of the HypCD complex and the HypCDE ternary complex: transient intermediate complexes during [NiFe] hydrogenase maturation. Structure 2012; 20: 2124–37.
46. Soboh B, Stripp S, Muhr E, Granich C, Braussemann M, Herzberg M, Heberle J, Sawers RG. [NiFe]-Hydrogenase maturation: isolation of a HypC-HypD complex carrying diatomic CO and CN(-) Ligands. FEBS Lett 2012; 586: 3882–7.
47. Soboh B, Stripp ST, Bielak C, Lindenstrauß U, Braussemann M, Javaid M, Hallensleben M, Granich C, Herzberg M, Heberle J, Sawers RG. The [NiFe]-hydrogenase accessory chaperones HypC and HybG of Escherichia coli are iron- and carbon dioxide-binding proteins. FEBS Lett 2013; 587: 2512–6.
48. Roseboom W, Blokesch M, Böck A, Albracht SPJ. The biosynthetic routes for carbon monoxide and cyanide in the Ni-Fe active site of hydrogenases are different. FEBS Lett 2005; 579: 469–72.
49. Imperial J, Rey L, Palacios JM, Ruiz-Argüeso T. HupK, a hydrogenase-ancillary protein from Rhizobium leguminosarum, shares structural motifs with the large subunit of NiFe hydrogenases and could be a scaffolding protein for hydrogenase metal cofactor assembly. Mol Microbiol 1993; 9: 1305–6.
50. Ludwig M, Schubert T, Zebger I, Wisitruangsakul N, Saggu M, Strack A, Lenz O, Hildebrandt P, Friedrich B. Concerted action of two novel auxiliary proteins in assembly of the active site in a membrane-bound [NiFe] hydrogenase. J Biol Chem 2009; 284: 2159–68.
51. Maróti J, Farkas A, Nagy IK, Maróti G, Kondorosi E, Rákhely G, Kovács KL. A second soluble Hox-type NiFe enzyme completes the hydrogenase set in Thiocapsa roseopersicina BBS. Appl Environ Microbiol 2010; 76: 5113–23.
52. Albareda M, Manyani H, Imperial J, Brito B, Ruiz-Argüeso T, Böck A, Palacios J-M. Dual role of HupF in the biosynthesis of [NiFe] hydrogenase in Rhizobium leguminosarum. BMC Microbiol 2012; 12: 256.
53. Maier T, Böck A. Generation of active [NiFe] hydrogenase in vitro from a nickel-free precursor form. Biochemistry 1996; 35: 10089–93.
54. Menon AL, Robson RL. In vivo and in vitro nickel-dependent processing of the [NiFe] hydrogenase in Azotobacter vinelandii. J Bacteriol 1994; 176: 291–5.
55. Rossmann R, Sauter M, Lottspeich F, Böck A. Maturation of the large subunit (HYCE) of Escherichia coli hydrogenase 3 requires nickel incorporation followed by C-terminal processing at Arg537. Eur J Biochem 1994; 220: 377–84.
56. Chan Chung KC, Zamble DB. Protein interactions and localization of the Escherichia coli accessory protein HypA during nickel insertion to [NiFe] hydrogenase. J Biol Chem 2011; 286: 43081–90.
57. Gasper R, Scrima A, Wittinghofer A. Structural insights into HypB, a GTP-binding protein that regulates metal binding. J Biol Chem 2006; 281: 27492–502.
58. Leach MR, Sandal S, Sun H, Zamble DB. Metal binding activity of the Escherichia coli hydrogenase maturation factor HypB. Biochemistry 2005; 44: 12229–38.
59. Chan Chung KC, Zamble DB. The Escherichia coli metal-binding chaperone SlyD interacts with the large subunit of [NiFe]-hydrogenase 3. FEBS Lett 2011; 585: 291–4.
60. Zhang JW, Butland G, Greenblatt JF, Emili A, Zamble DB. A role for SlyD in the Escherichia coli hydrogenase biosynthetic pathway. J Biol Chem 2005; 280: 4360–6.
61. Lutz S, Jacobi A, Schlensog V, Böhm R, Sawers RG, Böck A. Molecular characterization of an operon (hyp) necessary for the activity of the three hydrogenase isoenzymes in Escherichia coli. Mol Microbiol 1991; 5: 123–35.
62. Sydor AM, Liu J, Zamble DB. Effects of metal on the biochemical properties of Helicobacter pylori HypB, a maturation factor of [NiFe]-hydrogenase and urease. J Bacteriol 2011; 193: 1359–68.
63. Kleihues L, Lenz O, Bernhard M, Buhrke T, Friedrich B. The H_2 sensor of Ralstonia eutropha is a member of the subclass of regulatory [NiFe] hydrogenases. J Bacteriol 2000; 182: 2716–24.
64. Drapal N, Böck A. Interaction of the hydrogenase accessory protein HypC with HycE, the large subunit of Escherichia coli hydrogenase 3 during enzyme maturation. Biochemistry 1998; 37: 2941–8.
65. Kumarevel T, Tanaka T, Bessho Y, Shinkai A, Yokoyama S. Crystal structure of hydrogenase maturating endopeptidase HycI from Escherichia coli. Biochem Biophys Res Commun 2009; 389: 310–4.
66. Rossmann R, Maier T, Lottspeich F, Böck A. Characterisation of a protease from Escherichia coli involved in hydrogenase maturation. Eur J Biochem 1995; 227: 545–50.
67. Theodoratou E, Huber R, Böck A. [NiFe]-Hydrogenase maturation endopeptidase: structure and function. Biochem Soc Trans 2005; 33: 108–11.
68. Theodoratou E, Paschos A, Magalon A, Fritsche E, Huber R, Böck A. Nickel serves as a substrate recognition motif for the endopeptidase involved in hydrogenase maturation. Eur J Biochem 2000; 267: 1995–9.
69. Theodoratou E, Paschos A, Mintz-Weber, Böck A. Analysis of the cleavage site specificity of the endopeptidase involved in the maturation of the large subunit of hydrogenase 3 from Escherichia coli. Arch Microbiol 2000; 173: 110–6.
70. Fritsche E, Paschos A, Beisel HG, Böck A, Huber R. Crystal structure of the hydrogenase maturating endopeptidase HYBD from Escherichia coli. J Mol Biol 1999; 288: 989–98.
71. Magalon A, Blokesch M, Zehelein E, Böck A. Fidelity of metal insertion into hydrogenases. FEBS Lett 2001; 499: 73–6.
72. Pinske C, Sawers RG. Delivery of iron-sulfur clusters to the hydrogen-oxidizing [NiFe]-hydrogenases in Escherichia coli requires the A-type carrier proteins ErpA and IscA. PLoS One 2012; 7: e31755.
73. Page CC, Moser CC, Dutton PL. Mechanism for electron transfer within and between proteins. Curr Opin Chem Biol 2003; 7: 551–6.
74. Pinske C, Sawers RG. A-type carrier protein ErpA is essential for formation of an active formate-nitrate respiratory pathway in Escherichia coli K-12. J Bacteriol 2012; 194: 346–53.
75. Py B, Barras F. Building Fe-S proteins: bacterial strategies. Nat Rev Microbiol 2010; 8: 436–46.
76. Takahashi Y, Tokumoto U. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem 2002; 277: 28380–3.
77. Hu Y, Ribbe MW. Biosynthesis of nitrogenase FeMoco. Coord Chem Rev 2011; 255: 1218–24.

78. Jacobson MR, Cash VL, Weiss MC, Laird NF, Newton WE, Dean DR. Biochemical and genetic analysis of the nifUSVWZM cluster from Azotobacter vinelandii. Mol Gen Genet 1989; 219: 49–57.
79. Zheng L, White RH, Cash VL, Jack RF, Dean DR. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci USA 1993; 90: 2754–8.
80. Takahashi Y, Nakamura M. Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in Escherichia coli. J Biochem 1999; 126: 917–26.
81. Zheng L, Cash VL, Flint DH, Dean DR. Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii. J Biol Chem 1998; 273: 13264–72.
82. Fontecave M, Choudens SO de, Py B, Barras F. Mechanisms of iron-sulfur cluster assembly: the SUF machinery. J Biol Inorg Chem 2005; 10: 713–21.
83. Schwartz CJ, Giel JL, Patschkowski T, Luther C, Ruzicka FJ, Beinert H, Kiley PJ. IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins. Proc Natl Acad Sci USA 2001; 98: 14895–900.
84. Tokumoto U, Takahashi Y. Genetic analysis of the isc operon in Escherichia coli involved in the biogenesis of cellular iron-sulfur proteins. J Biochem 2001; 130: 63–71.
85. Shepard EM, Boyd ES, Broderick JB, Peters JW. Biosynthesis of complex iron-sulfur enzymes. Curr Opin Chem Biol 2011; 15: 319–27.
86. Adinolfi S, Iannuzzi C, Prischi F, Pastore C, Iametti S, Martin SR, Bonomi F, Pastore A. Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. Nat Struct Mol Biol 2009; 16: 390–6.
87. Ding H, Yang J, Coleman LC, Yeung S. Distinct iron binding property of two putative iron donors for the iron-sulfur cluster assembly: IscA and the bacterial frataxin ortholog CyaY under physiological and oxidative stress conditions. J Biol Chem 2007; 282: 7997–8004.
88. Layer G, Ollagnier-de Choudens S, Sanakis Y, Fontecave M. Iron-sulfur cluster biosynthesis: characterization of Escherichia coli CyaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. J Biol Chem 2006; 281: 16256–63.
89. Roche B, Aussel L, Ezraty B, Mandin P, Py B, Barras F. Iron/sulfur proteins biogenesis in prokaryotes: formation, regulation and diversity. Biochim Biophys Acta 2013; 1827: 455–69.
90. Vinella D, Brochier-Armanet C, Loiseau L, Talla E, Barras F. Iron-sulfur (Fe/S) protein biogenesis: phylogenomic and genetic studies of A-type carriers. PLoS Genet 2009; 5: e1000497.
91. Pinske C, Jaroschinsky M, Sawers RG. The levels of control exerted by the Isc iron-sulphur cluster system on the biosynthesis of the formate hydrogenlyase complex. Microbiology (Reading, Engl) 2013; 159: 1179–89.
92. Giel JL, Rodionov D, Liu M, Blattner FR, Kiley PJ. IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O_2-regulated genes in Escherichia coli. Mol Microbiol 2006; 60: 1058–75.
93. Nesbit AD, Fleischhacker AS, Teter SJ, Kiley PJ. ArcA and AppY antagonize IscR repression of hydrogenase-1 expression under anaerobic conditions revealing a novel mode of O_2 regulation of gene expression in Escherichia coli. J Bacteriol 2012; 194: 6892–9.
94. Oh Y-K, Raj SM, Jung GY, Park S. Current status of the metabolic engineering of microorganisms for biohydrogen production. Bioresour Technol 2011; 102: 8357–67.
95. Giel JL, Nesbit AD, Mettert EL, Fleischhacker AS, Wanta BT, Kiley PJ. Regulation of iron-sulphur cluster homeostasis through transcriptional control of the Isc pathway by [2Fe-2S]-IscR in Escherichia coli. Mol Microbiol 2013; 87: 478–92.
96. Rajagopalan S, Teter SJ, Zwart PH, Brennan RG, Phillips KJ, Kiley PJ. Studies of IscR reveal a unique mechanism for metal-dependent regulation of DNA binding specificity. Nat Struct Mol Biol 2013; 20: 740–7.
97. Mulder DW, Ortillo DO, Gardenghi DJ, Naumov AV, Ruebush SS, Szilagyi RK, Huynh B, Broderick JB, Peters JW. Activation of HydA(ΔEFG) requires a preformed [4Fe-4S] cluster. Biochemistry 2009; 48: 6240–8.
98. Mulder DW, Boyd ES, Sarma R, Lange RK, Endrizzi JA, Broderick JB, Peters JW. Stepwise [FeFe]-hydrogenase H-cluster assembly revealed in the structure of HydA(DeltaEFG). Nature 2010; 465: 248–51.
99. Soboh B, Kuhns M, Braussemann M, Waclawek M, Muhr E, Pierik AJ, Sawers RG. Evidence for an oxygen-sensitive iron-sulfur cluster in an immature large subunit species of Escherichia coli [NiFe]-hydrogenase 2. Biochem Biophys Res Commun 2012; 424: 158–63.
100. Palmer T, Berks BC. The twin-arginine translocation (Tat) protein export pathway. Nat Rev Microbiol 2012; 10: 483–96.
101. Sargent F. The twin-arginine transport system: moving folded proteins across membranes. Biochem Soc Trans 2007; 35: 835–47.
102. Menon NK, Robbins J, Wendt J, Shanmugam K, Przybyla A. Mutational analysis and characterization of the Escherichia coli hya operon, which encodes [NiFe] hydrogenase 1. J Bacteriol 1991; 173: 4851–61.
103. Jack RL, Buchanan G, Dubini A, Hatzixanthis K, Palmer T, Sargent F. Coordinating assembly and export of complex bacterial proteins. EMBO J 2004; 23: 3962–72.
104. Menon NK, Chatelus CY, Dervartanian M, Wendt JC, Shanmugam KT, Peck HD, Przybyla AE. Cloning, sequencing, and mutational analysis of the hyb operon encoding Escherichia coli hydrogenase 2. J Bacteriol 1994; 176: 4416–23.
105. Sargent F, Ballantine S, Rugman P, Palmer T, Boxer D. Reassignment of the gene encoding the Escherichia coli hydrogenase 2 small subunit-identification of a soluble precursor of the small subunit in a hypB mutant. Eur J Biochem 1998; 255: 746–54.
106. Dubini A, Pye R, Jack R, Palmer T, Sargent F. How bacteria get energy from hydrogen: a genetic analysis of periplasmic hydrogen oxidation in Escherichia coli. Int J Hydrogen Energy 2002; 27: 1413–20.
107. Manyani H, Rey L, Palacios JM, Imperial J, Ruiz-Argüeso T. Gene products of the hupGHIJ operon are involved in maturation of the iron-sulfur subunit of the [NiFe] hydrogenase from Rhizobium leguminosarum bv. viciae. J Bacteriol 2005; 187: 7018–26.
108. Schubert T, Lenz O, Krause E, Volkmer R, Friedrich B. Chaperones specific for the membrane-bound [NiFe]-hydrogenase interact with the Tat signal peptide of the small subunit precursor in Ralstonia eutropha H16. Mol Microbiol 2007; 66: 453–67.
109. Parish D, Benach J, Liu G, Singarapu KK, Xiao R, Acton T, Su M, Bansal S, Prestegard JH, Hunt J, Montelione GT, Szyperski T.

Protein chaperones Q8ZP25_SALTY from Salmonella typhimurium and HYAE_ECOLI from Escherichia coli exhibit thioredoxin-like structures despite lack of canonical thioredoxin active site sequence motif. J Struct Funct Genomics 2008; 9: 41–9.
110. Bernhard M, Schwartz E, Rietdorf J, Friedrich B. The Alcaligenes eutrophus membrane-bound hydrogenase gene locus encodes functions involved in maturation and electron transport coupling. J Bacteriol 1996; 178: 4522–29.
111. Casalot L, Rousset M. Maturation of the [NiFe] hydrogenases. Trends Microbiol 2001; 9: 228–37.
112. Fritsch J, Lenz O, Friedrich B. The maturation factors HoxR and HoxT contribute to oxygen tolerance of membrane-bound [NiFe] hydrogenase in Ralstonia eutropha H16. J Bacteriol 2011; 193: 2487–97.
113. Dubini A, Sargent F. Assembly of Tat-dependent [NiFe] hydrogenases: identification of precursor-binding accessory proteins. FEBS Lett 2003; 549: 141–6.
114. Hoppe A, Pandelia M-E, Gärtner W, Lubitz W. [Fe_4S_4]- and [Fe_3S_4]-cluster formation in synthetic peptides. Biochim Biophys Acta 2011; 1807: 1414–22.
115. Cvetkovic A, Menon AL, Thorgersen MP, Scott JW, Poole Ii FL, Jenney FE, Lancaster WA, Praissman JL, Shanmukh S, Vaccaro BJ, Trauger SA, Kalisiak E, Apon JV, Siuzdak G, Yannone SM, Tainer JA, Adams MWW. Microbial metalloproteomes are largely uncharacterized. Nature 2010; 466: 779–82.
116. Wu L, Mandrand-Berthelot M. Genetic and physiological characterization of new Escherichia coli mutants impaired in hydrogenase activity. Biochimie 1986; 68: 167–79.
117. Wu L, Mandrand-Berthelot M, Waugh R, Edmonds C, Holt S, Boxer D. Nickel deficiency gives rise to the defective hydrogenase phenotype of hydC and fnr mutants in Escherichia coli. Mol Microbiol 1989; 3: 1709–18.
118. Rowe JL, Starnes GL, Chivers PT. Complex transcriptional control links NikABCDE-dependent nickel transport with hydrogenase expression in Escherichia coli. J Bacteriol 2005; 187: 6317–23.
119. Mulrooney SB, Hausinger RP. Nickel uptake and utilization by microorganisms. FEMS Microbiol Rev 2003; 27: 239–61.
120. Brito B, Prieto R-I, Cabrera E, Mandrand-Berthelot M-A, Imperial J, Ruiz-Argüeso T, Palacios J-M. Rhizobium leguminosarum hupE encodes a nickel transporter required for hydrogenase activity. J Bacteriol 2010; 192: 925–35.
121. Eitinger T, Mandrand-Berthelot MA. Nickel transport systems in microorganisms. Arch Microbiol 2000; 173: 1–9.
122. Parkin A, Bowman L, Roessler MM, Davies RA, Palmer T, Armstrong FA, Sargent F. How Salmonella oxidises H_2 under aerobic conditions. FEBS Lett 2011; 586: 536–44.
123. Andrews S, Robinson A, Rodriguez-Quinones F. Bacterial iron homeostasis. FEMS Microbiol Rev 2003; 27: 215–37.
124. Cartron M, Maddocks S, Gillingham P, Craven C, Andrews S. Feo – transport of ferrous iron into bacteria. Biometals 2006; 19: 143–57.
125. Hantke K. Is the bacterial ferrous iron transporter FeoB a living fossil? Trends Microbiol 2003; 11: 192–5.
126. Hantke K. Regulation of ferric iron transport in Escherichia coli K12: isolation of a constitutive mutant. Mol Gen Genet 1981; 182: 288–92.
127. Hantke K. Members of the Fur protein family regulate iron and zinc transport in E. coli and characteristics of the Fur-regulated FhuF protein. J Mol Microbiol Biotechnol 2002; 4: 217–22.
128. Pinske C, Sawers RG. The role of the ferric-uptake regulator Fur and iron homeostasis in controlling levels of the [NiFe]-hydrogenases in Escherichia coli. Int J Hydrogen Energy 2010; 35: 8938–44.
129. Pinske C, Sawers RG. Iron restriction induces preferential down-regulation of H_2-consuming over H_2-evolving reactions during fermentative growth of Escherichia coli. BMC Microbiol 2011; 11: 196.
130. Martin W, Baross J, Kelley D, Russell MJ. Hydrothermal vents and the origin of life. Nat Rev Microbiol 2008; 6: 805–14.
131. Berggren G, Adamska A, Lambertz C, Simmons TR, Esselborn J, Atta M, Gambarelli S, Mouesca J-M, Reijerse E, Lubitz W, Happe T, Artero V, Fontecave M. Biomimetic assembly and activation of [FeFe]-hydrogenases. Nature 2013; 499: 66–9.
132. Esselborn J, Lambertz C, Adamska-Venkatesh A, Simmons T, Berggren G, Noth J, Siebel J, Hemschemeier A, Artero V, Reijerse E, Fontecave M, Lubitz W, Happe T. Spontaneous activation of [FeFe]-hydrogenases by an inorganic [2Fe] active site mimic. Nat Chem Biol 2013; 9: 607–9.
133. Hu Y, Ribbe MW. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. J Biol Chem 2013; 288: 13173–7.
134. McGuffee SR, Elcock AH. Diffusion, crowding & protein stability in a dynamic molecular model of the bacterial cytoplasm. PLoS Comput Biol 2010; 6: e1000694.
135. Liu W, Wacker D, Gati C, Han GW, James D, Wang D, Nelson G, Weierstall U, Katritch V, Barty A, Zatsepin NA, Li D, Messerschmidt M, Boutet S, Williams GJ, Koglin JE, Seibert MM, Wang C, Shah STA, Basu S, Fromme R, Kupitz C, Rendek KN, Grotjohann I, Fromme P, Kirian RA, Beyerlein KR, White TA, Chapman HN, Caffrey M, Spence JCH, Stevens RC, Cherezov V. Serial femtosecond crystallography of G protein-coupled receptors. Science 2013; 342: 1521–4.
136. Kilburn MR, Clode PL. Elemental and isotopic imaging of biological samples using NanoSIMS. Methods Mol Biol 2014; 1117: 733–55.
137. Rossmann R, Sawers RG, Böck A. Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. Mol Microbiol 1991; 5: 2807–14.
138. Bernhard M, Friedrich B, Siddiqui RA. Ralstonia eutropha TF93 is blocked in tat-mediated protein export. J Bacteriol 2000; 182: 581–8.
139. Pinske C, Krüger S, Soboh B, Ihling C, Kuhns M, Braussemann M, Jaroschinsky M, Sauer C, Sargent F, Sinz A, Sawers RG. Efficient electron transfer from hydrogen to benzyl viologen by the [NiFe]-hydrogenases of Escherichia coli is dependent on the coexpression of the iron-sulfur cluster-containing small subunit. Arch Microbiol 2011; 193: 893–903.

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