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Maturation of [NiFe]-hydrogenases in *Escherichia coli*

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Abstract Hydrogenases catalyze the reversible oxidation of dihydrogen. Catalysis occurs at bimetallic active sites that contain either nickel and iron or only iron and the nature of these active sites forms the basis of categorizing the enzymes into three classes, the [NiFe]-hydrogenases, the [FeFe]-hydrogenases and the iron sulfur cluster-free [Fe]-hydrogenases. The [NiFe]-hydrogenases and the [FeFe]-hydrogenases are unrelated at the amino acid sequence level but the active sites share the unusual feature of having diatomic ligands associated with the Fe atoms in the these enzymes. Combined structural and spectroscopic studies of [NiFe]-hydrogenases identified these diatomic ligands as CN^- and CO groups. Major advances in our understanding of the biosynthesis of these ligands have been achieved primarily through the study of the membrane-associated [NiFe]-hydrogenases of *Escherichia coli*. A complex biosynthetic machinery is involved in synthesis and attachment of these ligands to the iron atom, insertion of the $Fe(CN)_2CO$ group into the apo-hydrogenase, introduction of the nickel atom into the pre-formed active site and ensuring that the holoenzyme is correctly folded prior to delivery to the membrane. Although much remains to be uncovered regarding each of the individual biochemical steps on the pathway to synthesis of a fully functional enzyme, our understanding of the initial steps in CN^- synthesis have revealed that it is generated from carbamoyl phosphate. What is becoming increasingly clear is that the metabolic origins of the carbonyl group may be different.

Keywords Hydrogenase · CO and CN ligands · Metal centers · Cofactor biosynthesis · Nickel and iron

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Hydrogenases

A key future issue will be the replacement of fossil fuels with alternative energy sources. One energy economy that is currently being considered is based on dihydrogen (H_2). The advantages that hydrogen offers are its high calorific value and, if linked to oxygen reduction, it has zero carbon emission (Cammack et al. 2001; Melis and Happe 2001). Currently, the electrochemical activation of hydrogen utilizes platinum catalysts, which is an expensive process and in the long-term platinum is limited as a resource. The development of stable catalysts for hydrogen production and oxidation that are based on the bimetallic active sites of hydrogenases is one

alternative strategy being pursued to establish a robust hydrogen economy (Tard et al. 2005). In order to develop this economy we must first understand at the molecular level the structure and mechanism of these enzymes.

Hydrogenases catalyze the reversible oxidation of dihydrogen (Adams 1990; Nicolet et al. 2002 Sawers et al. 2004; Vignais and Colbeau 2004; Böck et al. 2006), and as such have the smallest substrate known in biology. The enzymes are highly sophisticated, have complex metal-based active site cofactors, which probably is the main reason for their generally unstable nature in the presence of molecular oxygen; although the hydrogenases of the aerobic bacterium *Ralstonia eutropha* are tolerant of oxygen (Burgdorf et al. 2005). Hydrogenases are found in archaea, many anaerobic bacteria and some lower eukarya, such as unicellular green algae, anaerobic ciliates and anaerobic fungi (Horner et al. 2002).

The nature of the metal in their respective active sites forms the basis of the distinction of hydrogenases into three classes. The first class comprises the [FeFe]-hydrogenases, which have a di-iron bimetallic site and they are mainly involved in hydrogen evolution. The [NiFe]-hydrogenases form the second class with an active site based on nickel and iron. Members of this class are generally hydrogen-oxidizing enzymes, although there is a sub-group of [NiFe]-hydrogenases which comprise multisubunit membrane-associated enzymes and these are hydrogen-evolving enzymes (Sawers et al. 2004). Members of these two classes, although unrelated at the primary structural level, are mainly heterodimeric enzymes; however, monomeric forms of [FeFe]-hydrogenases exist (Adams 1990; Peters 1999; Nicolet et al. 2002).

Both of these classes of hydrogenases have a similar overall architecture in which the active site is located within a large subunit and electrons are delivered to, or directed from, this center via a ''chain'' of iron–sulfur (Fe–S) centers located in the small subunit.

The third class of hydrogenase was identified comparatively recently (Zirngibl et al. 1992) and lacks Fe–S clusters. It is this feature of these enzymes that gives the class its name (Shima et al. 2004). Fe–S cluster-free hydrogenases (also referred to as [Fe]-hydrogenases: Armstrong and Albracht 2005) are found only in a small group of methanogenic archaea and function as hydrogen-forming methylenetetrahydromethanopterin dehydrogenases (Hmd). Although originally thought to lack any metal center, they have now been shown to have a novel cofactor based on a mononuclear iron core (Lyon et al. 2004b).

The structural characterization of first a soluble [NiFe]-hydrogenase from *Desulfovibrio gigas* (Volbeda et al. 1995) and then shortly thereafter [FeFe]-hydrogenases from *Clostridium pasteurianum* (Peters et al. 1998) and *Desulfovibrio desulfuricans* (Nicolet et al. 1999) identified the presence of diatomic ligands associated with the Fe atoms in these enzymes. Fourier transform infrared spectroscopy (FTIR) identified these diatomic ligands, initially in [NiFe]-hydrogenases, as 1 CO and 2 CN^- moieties (Pierik et al. 1999). [FeFe]-hydrogenases have five diatomic ligands coordinated to the active site metals, with each Fe atom coordinating 1 CN^- and 1 CO and an additional CO moiety bridging the two Fe atoms (Peters et al. 1998; Pierik et al. 1998; Nicolet et al. 1999). Recently, FTIR spectroscopy has also indicated the presence of 2 CO ligands to the Fe atom of the unique cofactor in Hmd (Lyon et al. 2004a).

The *E. coli* genome encodes four [NiFe]-hydrogenases

Although *E. coli* had been known for a long time to express hydrogenase activity (Stephenson and Stickland 1931; Adams and Hall 1979) when grown anaerobically, it was not until the mid-1980s that it was demonstrated that this is due to the activities of three immunologically distinct enzymes (see Fig. 1), which are expressed under different anaerobic growth conditions (Ballantine and Boxer 1985; Sawers et al. 1985). All three of these enzymes are [NiFe]-hydrogenases (Ballantine and Boxer 1986; Rossmann et al. 1994; Maier and Böck 1996; Sawers and Boxer 1986). Hydrogenase 1 (Hyd-1) is the most abundant hydrogenase in *E. coli* and is synthesized primarily under fermentative conditions, catalyzing hydrogen oxidation, and it has been suggested that this enzyme

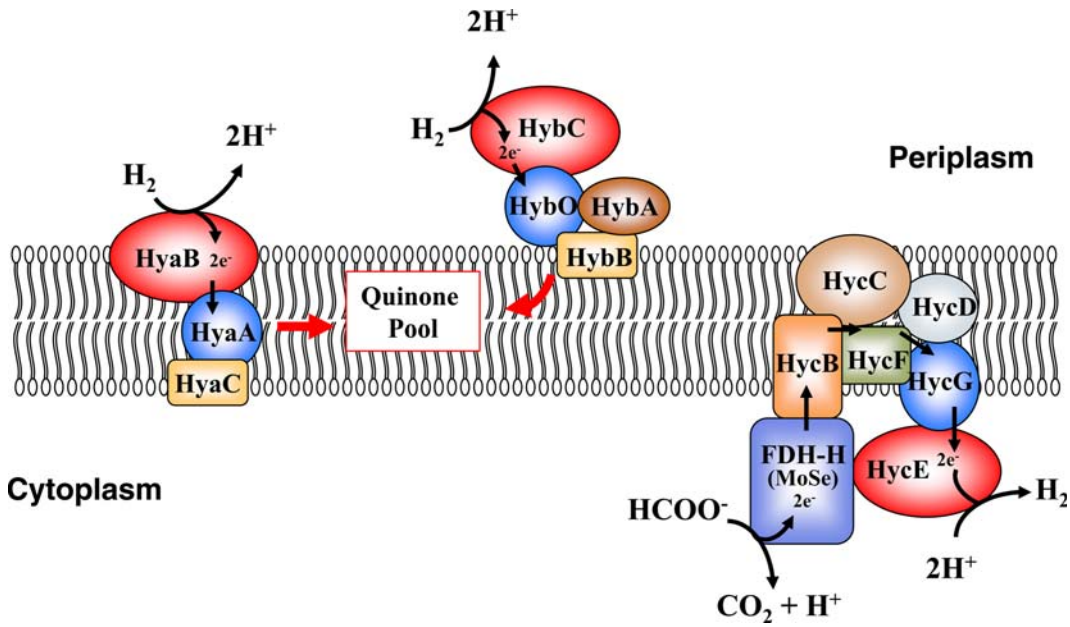

Fig. 1 Schematic representation of the localization of [NiFe]-hydrogenases 1, 2 and 3 (the FHL complex) in the cytoplasmic membrane of *E. coli*. Note that Hya proteins form Hyd-1, Hyb proteins form Hyd-2 and Hyc proteins form the formate hydrogenlyase (FHL) complex, which includes the molybdoseleno-protein formate dehydrogenase H (FDH-H)

is involved in hydrogen recycling (Sawers et al. 1985). Hyd-2 is synthesized at high levels when cells are grown with glycerol and fumarate or hydrogen and fumarate and is responsible for energy generation under these conditions. The third hydrogenase, Hyd-3, forms part of a large, unstable and biochemically intractable multi-enzyme formate hydrogenlyase (FHL) complex that catalyzes the disproportionation of formate into carbon dioxide and hydrogen (Rossmann et al. 1991). The enzyme complex comprises a molybdo-selenium formate dehydrogenase, encoded by the *fdhF* gene (Zinoni et al. 1986), and six polypeptides encoded by the *hyc* operon (Böhm et al. 1990; Sauter et al. 1992). Expression of both the *fdhF* gene and the *hyc* operon is tightly coordinated and totally dependent on formate and acidification of the growth medium (Rossmann et al. 1991), conditions that prevail when *E. coli* grows by mixed acid fermentation.

Hyd-3 is a member of a recently defined subclass of [NiFe]-hydrogensases that have been termed energy-converting hydrogenases (Ech) (Hedderich and Forzi 2005). Family members are membrane-associated and between five and six subunits of the core protein complex share significant amino acid sequence similarity with subunits of the catalytic core of the NADH:quinone oxidoreductase (complex I), suggesting that they are involved in energy metabolism. Although a possible role of the FHL complex in energy conservation has been speculated upon (Sauter et al. 1992), to date no clear evidence for this has been demonstrated. At low partial pressures of hydrogen in vivo this reaction is exergonic (~ –20 kJ mol^{-1}).

Just prior to the public release of the *E. coli* genome sequence, an operon encoding a putative fourth hydrogenase, *hyf*, was identified (Andrews et al. 1997). The *hyf* operon encodes homologues of all the Hyc components of the FHL complex together with an additional three membrane components that share sequence similarity with complex I components. Based on these findings it has been proposed that the Hyf complex represents a putative proton-translocating FHL-2 complex (Andrews et al. 1997); however, expression of the *hyf* operon is very weak (Skibinski et al.

2002; Self et al. 2004) and conditions under which this system is synthesized in vivo have yet to be established.

Hyd-1 and Hyd-2 have their respective active sites oriented towards the periplasm, while Hyd-3, and presumably Hyd-4 also, has a cytoplasmic localization (Fig. 1; Sargent et al. 1998; Sawers et al. 2004). Although Hyd-1 and Hyd-2 have a similar modular structure, their topology in the membrane is very different. Early labeling studies suggested a trans-membrane topology for hydrogenase (Graham 1981), but this was before the existence of multiple enzymes was recognized. Based on our current understanding, it would seem that those labeling studies identified Hyd-1. Indeed, this trimeric enzyme can only be released from the membrane upon detergent treatment. Hyd-2, on the other hand, is a tetrameric enzyme in which HybC is the large subunit, HybO the small subunit, which includes a membrane anchor, and this dimer associates with the ''poly-ferredoxin'' subunit HybA and an integral membrane protein HybB. Hyd-2 can be released in a soluble, active form by limited proteolytic treatment with trypsin (Ballantine and Boxer 1985; Sargent et al. 1998), suggesting that it is anchored in the membrane by a short peptide. What significance these different membrane topologies, particularly that of Hyd-1, have for the functions of these related enzymes is still unclear.

Active site structure of [NiFe]-hydrogenases

The combined findings of structural and spectroscopic analyses have provided an accurate picture of the bimetallic center of [NiFe]-hydrogenases (Volbeda et al. 1995; DerVartanian et al. 1996; Armstrong and Albracht 2005; Volbeda et al. 2005). The nickel atom is in the Ni^{2+} or Ni^{3+} oxidation states, and is coordinated by four Cys thiolates that are highly conserved within this class of hydrogenase (Fig. 2). Two of these cysteinyl residues also coordinate the Fe atom. A bridging ligand, thought to be either a hydroxide or a hydroperoxide in the *D. fructosovorans* enzyme (Volbeda et al. 2005), possibly fills the coordination site normally occupied by H_2.

The Fe atom is of interest because it has three diatomic ligands coordinated to it. These ligands stabilize the Fe in a low oxidation and spin state (Fe^{2+}) and presumably facilitate the activation of hydrogen at the Ni atom. The existence of these ligands, when first discovered, was unprecedented in biological systems and the question arose as to how these toxic compounds are synthesized, compartmentalized from the rest of the cellular metabolic processes and incorporated into the bimetallic cofactor.

Identification of accessory gene products involved in active site synthesis and enzyme maturation

Early mutagenesis studies with *E. coli* resulted in the isolation of various mutants with defects in hydrogen metabolism (reviewed in Sawers et al. 2004). Because it was initially believed that *E. coli* had only one hydrogenase, mutants lacking total hydrogenase activity had lesions in genes whose products were not structural components of the enzymes but in proteins necessary for common pathways in the biosynthesis of all hydrogenase enzymes. Most of the genes affected by these mutations were in one particular region of the chromosome and cloning and sequencing of this region (Böhm et al. 1990; Lutz et al. 1991; Jacobi et al. 1992) identified two divergently transcribed operons (Fig. 3). The *hyc* operon encodes mainly structural components of the FHL complex, while the *hyp* (hydrogen pleiotropy) operon encodes five proteins, two of which, HypA and HypC, are specific for the maturation of Hyd-3, while the remaining three proteins, HypB, D, and E are essential for maturation of all hydrogenases. Orthologues of HypA and HypC are the HybF and HybG proteins, respectively, encoded as part of the *hyb* operon specifying Hyd-2, and they are specific for maturation of Hyd-1 and Hyd-2 (Menon et al. 1994).

A further gene *hypF* is located downstream of the *hyc* operon, and when mutated also results in a complete lack of hydrogenase activity (Maier et al. 1996). The lesions in some of the *hyp* gene mutants (*hypA, hypB*) can be overcome by supplementation with high concentrations of nickel (Waugh and Boxer 1986; Maier et al.

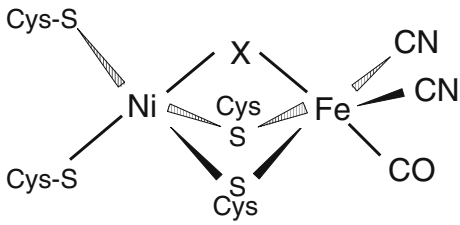

Fig. 2 Diagramatic representation of the Ni–Fe active site cofactor of [NiFe]-hydrogenases. Cys-S represents thiolate ligands derived from the polypetide backbone. X is likely to be either a hydroxide or hydroperoxide (Volbeda et al. 2005)

1993; Hube et al. 2002), indicating that they have a role in nickel insertion.

More recently mutations in the *carAB* operon were also found to abolish hydrogenase activity (Paschos et al. 2001). The operon encodes carbamoyl phosphate synthase, and carbamoyl phosphate is a precursor of the cyano ligand (see below). Mutations mapping to the *pyrA* locus (synonymous with *car*) were originally identified in the 1980s in *Salmonella enterica* serovar typhimurium (Barrett et al. 1984).

Together with information provided by the deduced amino acid sequences of the various maturation enzymes, a key development in the elucidation of the putative biochemical functions of the Hyp proteins in hydrogenase maturation was the ability to create and study systematic in-frame deletion mutations. This not only allowed the verification of previously observed phenotypes but it aided the identification of further gene products necessary for the maturation and assembly of active, membrane-associated enzymes. During the study of the *hyc* operon it was noted that mutations in the final gene of the operon *hycI* resulted in a FHL^- phenotype but the synthesis of Hyd-1 and Hyd-2 was unaffected (Rossmann et al. 1994). Close examination of the large subunit, HycE, of Hyd-3 revealed that it was present in a larger precursor form. Further work demonstrated that the processing event involves the removal of a small C-terminal polypeptide, which takes place only after nickel insertion. The cleavage site is located two amino acid residues C-terminal to the fourth conserved cysteinyl residue that coordinates the active site cofactor (Rossmann et al. 1994, 1995). Comparable studies with Hyd-1 and Hyd-2 revealed that they also each have a sequence-specific protease (HyaD and HybD) encoded within their respective structural gene operons (Menon et al. 1991, 1994).

Finally, the active sites of both Hyd-1 and Hyd-2 face the periplasm and the ''matured'', active

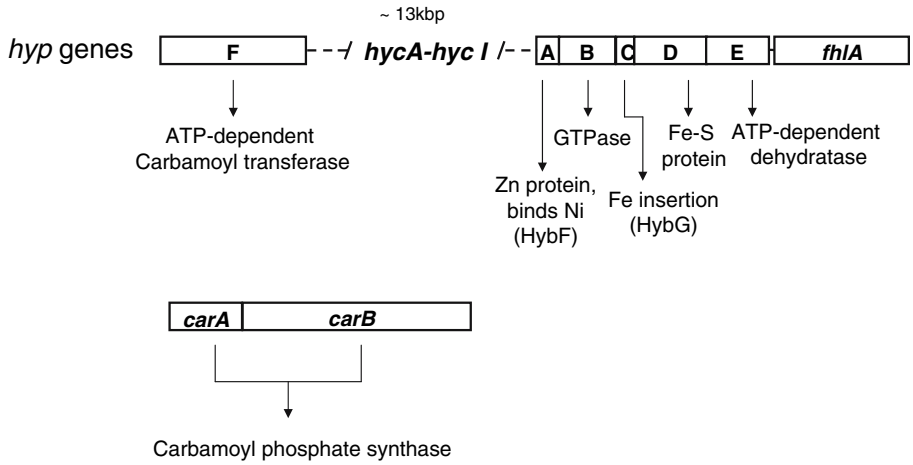

Fig. 3 Organization of the genes and operons whose products have been shown to be required for biosynthesis of the active site cofactor of [NiFe]-hydrogenases in *E. coli*. The biochemical functions of the respective gene products are described below each gene. The *fhlA* gene encodes a transcriptional regulator necessary for the formate-dependent expression of the *hyc* operon and *fdhF* gene, which encode the structural components of the FHL complex (Rossmann et al. 1991). In the interest of clarity the individual genes of the *hycA-I* operon, which is divergently transcribed from the *hyp* operon, are not drawn in full

site-bearing enzymes both must be transported across the cytoplasmic membrane by the TAT (twin-arginine transport) pathway, which transports mature, folded proteins (Palmer et al. 2005). Early deletion studies identified three further genes *hyaE* and *hyaF* and *hybE*, essential for formation of the respective active, membrane-associated hydrogenases (Menon et al. 1991, 1994). Subsequent work (Dubini and Sargent 2004) has shown that the HyaE and HybE proteins interact with the respective signal peptides of Hyd-1 and Hyd-2 subunits and they have been proposed to exert ''proof-reading'' functions that prevent Tat-dependent transport of improperly folded proteins. It is possible that the HyaF protein also has a specific role in determining the membrane-integral orientation of Hyd-1.

The maturation pathway: CN ligand synthesis

Identification of carbamoyl phosphate as a precursor of the cyano ligands of [NiFe]-hydrogenases was a watershed, because it immediately gave credence to observation that HypF shares a sequence motif with *O*-carbamoyl transferases and suggested a testable hypothesis for the formation of CN^- (Paschos et al. 2002). HypF also shares sequence similarity with acyl phosphatases, which substantiated this hypothesis. Purified HypF hydrolyzes carbamoyl phosphate, but in the presence of ATP and purified HypE it catalyzes the transfer of the carbamoyl to the C-terminal cysteinyl residue of HypE generating a thio-carboxamide, with the concomitant generation of AMP and pyrophosphate (Paschos et al. 2002). This latter observation suggests the possible involvement of a carbamoyladenylate intermediate. In a further ATP-dependent dehydratase reaction HypE activates the oxygen of the carboxamide and after phosphorylation/dephosphorylation the moiety is converted to a thiocyanate (Blokesch et al. 2004b; Reissmann et al. 2003). These steps are outlined in the scheme presented in Fig. 4.

The subsequent steps are speculative with regard to which protein the cyano ligand is transferred and the mechanism underlying this transfer. First, thiocyanates are known to be good donors of CN^- to iron (cited in Reissmann et al. 2003), therefore it is likely that HypE-thiocyanate is the cyano donor to the next protein on the scaffold. It was noted that in mutants unable to synthesize carbamoyl phosphate a complex between the HypC and HypD proteins was observed (Blokesch and Böck 2002). Resolution of this complex could be achieved by addition of citrulline, which can serve as a precursor of carbamoyl phosphate, suggesting that they accept the CN^- ligand from HypE. More recent studies have shown that the HypC–HypD complex is an intermediate in the cyano group transfer (Blokesch et al. 2004a). Furthermore, HypC has been shown to form a complex with the cofactor-free from of the large subunit of Hyd-3 (Drapal and Böck 1998; Magalon and Böck 2000a), suggesting that HypC might deliver the $Fe(CN)_2(CO)$ group into the active site of the apoenzyme.

Several features are noteworthy about the HypC and HypD proteins. First, HypC has an N-terminal cysteinyl residue that is essential for its interaction with HypD and the large subunit of Hyd-3 (Blokesch and Böck 2002; Magalon and Böck 2000a). This suggest that at some stage on the maturation pathway, this cysteinyl residue might act as acceptor for the modified Fe atom. The HypC–HypD complex is able to interact with HypE and HypF (Blokesch et al. 2004a), and therefore in principle either protein could accept the CN^- moiety directly from HypE. Notably, a recent study (Butland et al. 2006) has demonstrated that the HypC homologue HybG, which is required for maturation of Hyd-1 and Hyd-2 (Blokesch et al. 2001), forms a complex with HypD, HypE and the large subunit precursors of Hyd-1 and Hyd-2. HypD also has a Fe–S cluster that exhibits unusual redox chemistry (Blokesch et al. 2004a; Roseboom et al. 2005), which might be a reflection of its atypical coordination to the protein. In a recent study (Blokesch and Böck 2006) seven essential cysteinyl residues in three distinct motifs were identified, which are essential for the function of the protein. This finding suggests that HypD might have a further as yet unidentified cofactor associated with it, or that these cysteinyl residues play an important role in the interaction with HypC, the stabilization of the [4Fe–4S] cluster in HypD and the tuning of the

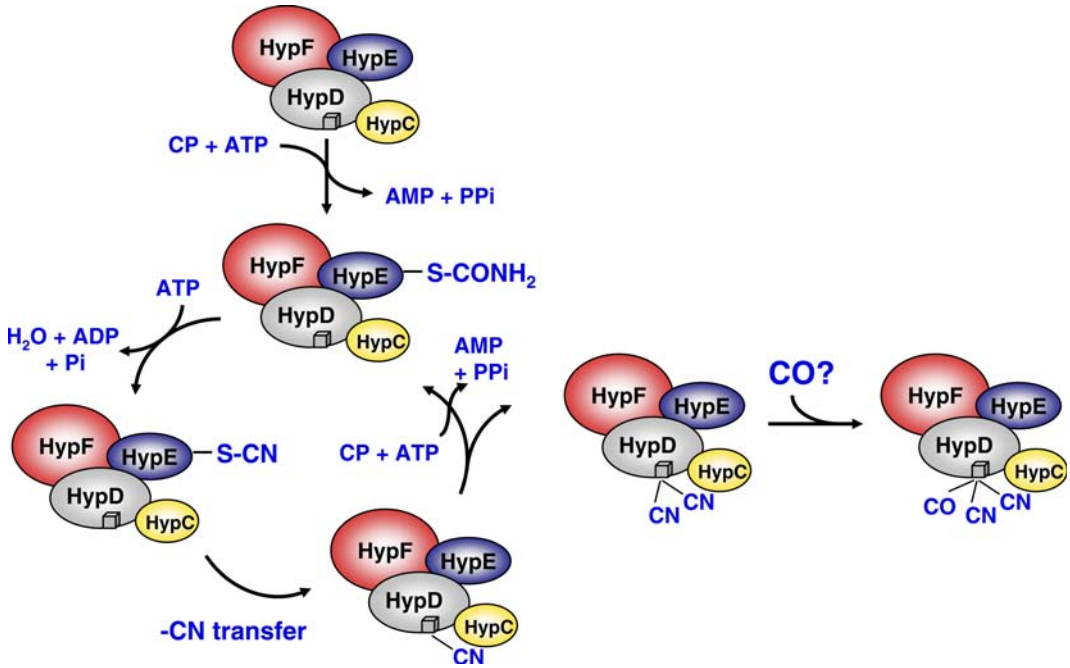

Fig. 4 Pathway and proteins of cyano and carbonyl ligand biosynthesis. The question mark on the right of the Figure indicates that it is unclear whether the carbonyl is added prior or subsequent to CN incorporation. The small cube within HypD signifies a [4Fe–4S] cluster. Abbreviations: CP, carbamoyl phosphate; PPi, pyrophosphate; CO, carbonyl ligand; CN, cyano ligand

redox potential of the cluster. Since the transfer of the CN^- to the Fe atom is an electrophilic substitution reaction, it is possible that HypD directly transfers the cyano group to a Fe atom coordinated to the N-terminal cysteinyl of HypC. Alternatively, one of the Fe atoms of the [4Fe–4S] cluster of HypD might serve as the scaffold for the cyanation reaction. Structural resolution of an intermediate complex of HypC and HypD in which one or two cyano ligands are attached will prove crucial in resolving this question.

The maturation pathway: CO ligand synthesis

The metabolic origin of the carbonyl ligand to the Fe in [NiFe]-hydrogenases is unknown. It is at least theoretically possible that CO could also be generated from carbamoyl phosphate and this was proposed in early studies (Reissmann et al. 2003). Initial transfer of a third cyano group to the Fe atom, followed by hydrolysis to the carbonyl would be one possibility (Jiang et al. 2001; Reissmann et al. 2003). Alternatively, CO could be synthesized directly at HypE or HypD. Direct transfer of the carbamoyl to the Fe followed by deamination of the Fe-carboxamido complex at the HypCD complex has been ruled out (Roseboom et al. 2005).

The spectroscopic identification of Fe–CN and Fe–CO complexes in hydrogenases using FTIR has provided an invaluable tool in the analysis of these diatomic ligands (Van der Spek et al. 1996). The nature of the polar triple bond to the metal coordinated within the protein environment yields unique absorption bands in the infrared spectral region of 2,100–1,850 cm^{-1}. Because these signals are in an isolated region of the spectrum this facilitates labeling studies using ^{13}C compounds. The results of a key study involving labeling studies with the [NiFe]-hydrogenase from *Allochromatium vinosum* suggest that the metabolic origins of the CN^- and CO ligands to the Fe atom are different (Roseboom et al. 2005). Using $NaH^{13}CO_3$ and ^{13}C-acetate labeled either at the C1 or C2 position the authors provided

strong evidence to indicate that, at least in *A. vinosum*, the CN^- and CO^- moieties derive from different sources. Significantly, labeling of the CO ligand to the iron in the active site of the hydrogenase was only observed when the C1 carbon of acetate was labeled (Roseboom et al. 2005), suggesting that, at least in *A. vinosum*, CO can be derived from the carboxylate carbon of acetate, analogous to an acetyl-CoA synthase-CO dehydrogenase-driven carboxyl-CO exchange reaction (Ragsdale 2004). We have also observed that the CN and CO ligands for Hyd-2 of *E. coli* are derived from different metabolic sources and independently confirmed that CN^- is derived from carbamoyl phosphate (L. Forzi, P. Hellwig, A. Böck, R. K. Thauer and R. G. Sawers, unpublished results). These findings might suggest a new reaction involving one of the Hyp protein components.

Possible metabolic origins of CO

In the scheme shown in Fig. 4, the incorporation of CO is depicted as occurring post CN^- addition to the Fe. It could conceivably also occur prior to CN addition, however, it has been observed for the isolated iron–molybdenum cofactor (Fe-MoCo) of nitrogenase that binding of CN^- synergistically enhances CO binding to the complex, while in the absence of CN^-, CO is unreactive (Pickett et al. 2004). This probably occurs by CN^- stabilizing a low-spin, oxidized form of the cofactor. This observation may be of relevance to the order of diatomic ligand addition to the Fe atom of hydrogenases.

The fact that our current data suggest that the metabolic origins of CO and CN^- differ implies either further biochemical functions associated with one or more of the Hyp proteins or a further protein(s) is involved in the incorporation of CO onto the Fe atom. It is also clear that in the steps prior to final addition of the CO ligand, a different system of enzymes is involved in generation of CO.

Which pathways could contribute to CO generation in *E. coli*? Two aspects must be noted when considering the possible sources of CO in the context of a diatomic ligand for [NiFe]-hydrogenases. First, it is important to note that the CO_2/CO redox couple has a reduction potential of –524 mV at pH 7.0 (Thauer 1990) and consequently is significantly more reducing than the H^+/H_2 redox couple of –414 mV at pH 7.0 and 1 atm partial pressure. This means that generation of CO will require input of energy. Second, the pathway of CO generation is likely to be common to most, if not all, bacteria that synthesize [NiFe]-hydrogenases.

Possible metabolic sources of CO are shown in Fig. 5. Formate could formally act as an indirect precursor of CO, but would require activation (CO_2/formate redox couple of –432 mV at pH 7.0) to the level of N^{10}-formyl-THF. *Escherichia coli*, however, does not have a formyl-THF synthetase (Mathews 1996), and therefore a new enzyme activity would need to be invoked that would activate formate to the level of CO.

N^{10}-formyl-THF is a metabolic intermediate common to all bacteria and is the one-carbon donor in purine biosynthesis and the formylation of methionine required for translation initiation. N^{10}-formyl-THF is interconvertible with other THF derivatives and is generated from methylene-THF by the action of methylene-THF dehydrogenase–methenyl-THF cyclohydrolase. Thus, ultimately, it is derived from serine or glycine (Mathews 1996). Notably, glycine has also recently been hypothesized as the putative metabolic source of the CN^- and CO ligands in the active site of [FeFe]-hydrogenases (Peters et al. 2005), which appear to have evolved an accessory system for active site synthesis that is based on SAM-radical biochemistry (Rubach et al. 2005; Böck et al. 2006).

A potential direct source of CO would be from heme degradation by the action of heme oxygenases (Tenhunen et al. 1969). However, formation of CO is known to occur only during aerobic growth.

It is also possible that CO could be generated from the carboxylate carbon of acetyl-CoA by the reversible acetyl-CoA synthase-CO dehydrogenase enzyme complex, found in acetogenic bacteria (Ragsdale 2004), and postulated recently to be a potential route of CO generation in *Allochromatium vinosum* (Roseboom et al. 2005). However, the *E. coli* genome does not

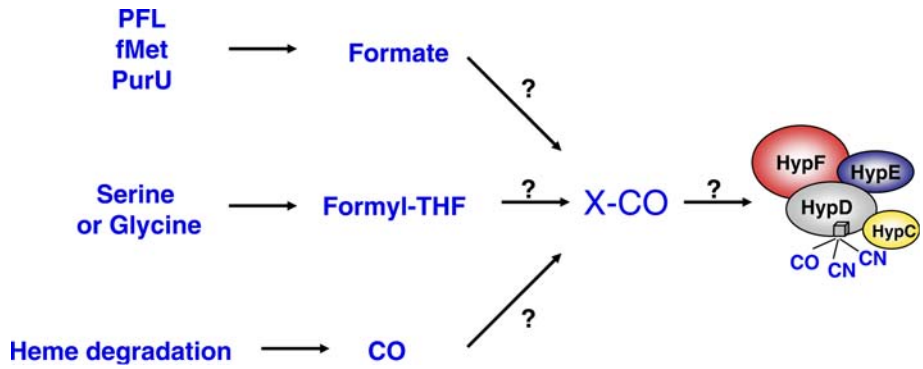

Fig. 5 Possible metabolic origins of carbonyl ligand synthesis. Abbreviations: fMet, formyl-methionine; formyl-THF, N^{10} formyl-tetrahydrofolate; PFL, pyruvate formate-lyase; PurU, N^{10} formyl-tetrahydrofolate hydrolase (Zalkin 1997)

encode such an enzyme complex and thus this pathway would not fulfill the requirement of a common pathway for CO generation amongst bacteria that synthesize [NiFe]-hydrogenases.

It is also conceivable that, since only small amounts of CO are necessary for synthesis of the diatomic ligand of hydrogenases, it could be generated from more than one source, depending upon the organism.

Nickel insertion

Evidence from nickel depletion studies performed with nickel transport mutants suggests that nickel is inserted after introduction of $Fe(CN)_2(CO)$ into the active site (Maier and Böck 1996). Moreover, in *hyp* gene mutants the large subunit precursor does not contain nickel (Maier et al. 1993; Magalon and Böck 2000b). Nickel is delivered to the active site by the nickel-binding zinc metalloenzyme HypA, which is specific for Hyd-3 (Jacobi et al. 1992; Atanassova and Zamble 2005), or its homologue HybF, which specifically delivers nickel to Hyd-1 and Hyd-2 (Hube et al. 2002; Blokesch et al. 2004c). The other component required for nickel insertion is the HypB protein (Fig. 6), which is a metal- and GTP-binding and hydrolysis protein (Maier et al. 1993; Leach et al. 2005; Gasper et al. 2006). Remarkably, addition of high nickel concentrations to the medium of mutants lacking these components results in mature, nickel-containing hydrogenases (Waugh and Boxer 1986; Hube et al. 2002), indicating that these proteins function to improve the kinetics or indeed the fidelity of nickel insertion. The role of HypB might be as a switch (Gasper et al. 2006), which, upon GTP hydrolysis, releases the complex from the hydrogenase large subunit upon successful nickel insertion (Gasper et al. 2006).

Recently, the peptidyl-prolyl *cis*/*trans* isomerase SlyD was also shown to improve the kinetics of nickel insertion (Zhang et al. 2005). SlyD interacts specifically with HypB and possibly aids either proper folding of the large subunit after nickel incorporation, or assembly of the protein complex required for nickel insertion.

Active site closure: the endoproteolytic cleavage reaction

Cleavage of the C-terminal peptide on the large subunit precursor is a substrate-specific event and in the case of the *E. coli* hydrogenases, each has its own specific protease, encoded within the structural gene operon. The proteases HyaD, HybD and HycI perform the final maturation steps of Hyd-1, Hyd-2 and Hyd-3, respectively (Menon et al. 1991, 1994; Rossmann et al. 1995). It is unclear which protease is responsible for the maturation of HyfG of Hyd-4.

Peptide cleavage occurs only after the insertion of nickel into the active site has taken place (Theodoratou et al. 2000) (Fig. 6). Although purified HycI and HybD lack a metal (Fritsche et al. 1999; Theodoratou et al. 2000), HycI has nevertheless been shown to have proteolytic

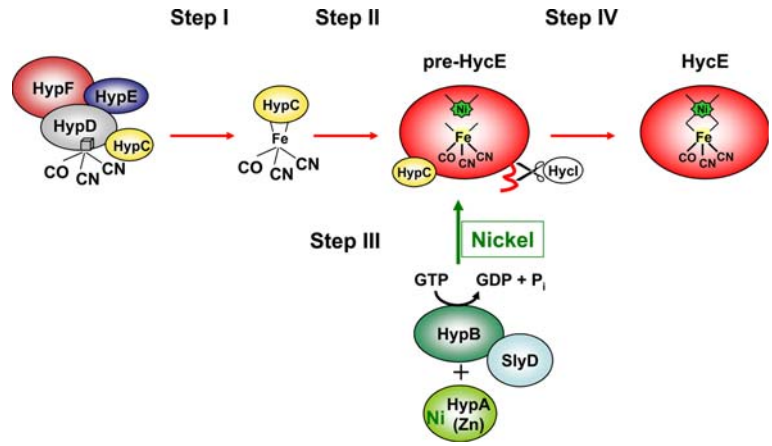

Fig. 6 Model for the completion of active site synthesis of [NiFe]-hydrogenase 3 in *E. coli*. Step I, the preformed $Fe(CN)_2CO$ group is transferred to HypC. Step II, HypC delivers the $Fe(CN)_2CO$ group to the hydrogenase large subunit precursor, pre-HycE. Step III, The HypA–HypB–SlyD complex delivers nickel to the Fe-containing precursor. Step IV, the HycE-specific protease (HycI) cleaves the C-terminal peptide to complete active site closure

activity in vitro (Rossmann et al. 1995). The structure of HybD has been resolved with cadmium bound to the protein (Fritsche et al. 1999). The metal is liganded by four amino acids and a water molecule and mutagenesis studies have shown that exchange of these amino acids for dissimilar ones results in an inactive protein (Theodoratou et al. 2000); however residual activity is retained when the exchanges are made with chemically similar amino acids. Taken together, these studies suggest that these residues constitute the recognition site for nickel, bound within the active site. Whether this occurs while the large subunit precursor is in complex with the HypA/HybD and HypB is unclear.

Due to the conservation of amino acid sequence around the cleavage site, and the fact that excessive truncation of the C-terminal peptide impedes processing, it has been proposed (Sawers et al. 2004) that the C-terminal extension acts as an endo-chaperone, which maintains the active site in a particular conformation allowing introduction of the metal center. Gel electrophoretic analysis of the enzyme pre- and post-cleavage indicates that a large conformational change must occur upon removal of the peptide and it is thought that this event ''seals'' the active site within the protein and might even signal interaction with the small subunit (Magalon and Böck 2000b).

Membrane insertion

Although all three characterized [NiFe]-hydrogenases in *E. coli* are membrane bound, only Hyd-1 and Hyd-2 have their active sites exposed to the periplasm. Since the machinery for the biosynthesis and insertion of the Ni–Fe cofactor is localized within the cytoplasm, this indicates that the holoenzymes must be exported as ''near-complete'', folded proteins and this can only be achieved by the Tat transport machine (Dubini and Sargent 2004; Palmer et al. 2005). The precursor β-subunits of the hydrogenases possess a cleavable signal peptide bearing the twin arginine motif SRRxFLK, which directs the folded $\alpha\beta$ HybCO dimer, including all cofactors, to the Tat translocase (Dubini and Sargent 2004). The proof-reading step (Jack et al. 2004) required to ensure that only the folded dimeric protein is presented to, and allowed to engage with, the Tat translocase is performed by the specific chaperone HybE (Dubini and Sargent 2004). HybE also interacts with the precursor form of the HybC α-subunit. The corresponding HyaE chaperone interacts with Hyd-1 small subunit precursor HyaA (Dubini and Sargent 2004) and two-hybrid analyses also indicated that these chaperones probably form minimally dimer. Data also suggest that there is possibly some cross-talk between the hydrogenase assembly systems. This latter finding

is congruent with assembly of these complex enzymes occurring in large multiprotein complexes and indeed might suggest that the chaperones are associated with the Ni–Fe cofactor biosynthetic machinery. The molecular mechanisms underlying the proof-reading steps putatively performed by HyaE and HybE preventing premature access to the Tat machine are currently unclear.

Perspectives

A key objective in future research on the biosynthesis of [NiFe]-hydrogenases will be the elucidation of the metabolic origin of the CO ligand to the active site Fe. *E. coli* offers distinct advantages to resolve this problem, because the hydrogenases of this organism are well-characterized and readily amenable to biochemical analysis. Perhaps most important is the genetic tractability of *E. coli*, which has facilitated the elucidation of the biosynthetic route of CN-ligand synthesis. Genetic studies using defined, in-frame deletions in genes whose products are involved in serine, glycine and one-carbon metabolism, in combination with specific labeling experiments should help resolve the route of CO ligand generation. Clearly, whatever the origin of the CO, a new biochemical activity is either associated with one of the already characterized Hyp proteins, or a yet undiscovered enzyme is required to synthesize and transfer the CO to the Fe atom.

A further key objective will be the structural elucidation of the accessory hydrogenase maturation machinery. Again, the *E. coli* system offers distinct advantages in the initial structural analysis of the multienzyme complex(es) involved in this process. Structural resolution of these enzyme complexes might also shed light on the mechanisms underlying the individual components and, in particular, will be crucial in answering the important questions regarding CN^--ligand transfer within the complex and whether HypD or HypC form the scaffold for $Fe(CN)_2CO$ group synthesis. Ultimately, reconstitution of the complete biochemical pathway of active site synthesis in vitro will establish the validity of the in vivo findings.

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