

# Cyanide and Carbon Monoxide Ligand Formation in Hydrogenase Biosynthesis

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Carbon monoxide (CO) and cyanide (CN<sup>−</sup>) can act as potent inhibitors of enzymes necessary for primary biochemical processes, however they also play important roles in biological systems. Well-studied cases include CN<sup>−</sup> biosynthesis in plants to act as a defense against herbivores and pathogens, CN<sup>−</sup> biosynthesis in certain species of bacteria to remove excess glycine, and CO biosynthesis by microbes for energy metabolism in the Wood–Ljungdahl pathway. The utilization of CO and CN<sup>−</sup> as essential metal ligands in biology is even more limited, with the only known examples being at the active sites of hydrogenase enzymes. This class of enzymes catalyzes the reversible oxidation of hydrogen, a reaction that in biology appears to be entirely dependent on the presence of CO and/or CN<sup>−</sup> ligands. To date, synthetic mimics

of hydrogenase active sites have not reproduced hydrogen production rates observed in some hydrogenases; it is thus of considerable interest to understand how biology has solved the intriguing problem of biosynthesizing efficient hydrogen catalysts. Of the hydrogenase enzymes discussed herein, recent advances in the [FeFe]-hydrogenase family has provided important insights into the synthesis of the CO and CN<sup>−</sup> ligands for its active site (H-cluster). Biosynthesis of the complex [FeFe]-hydrogenase active site requires only three iron–sulfur cluster-containing maturation proteins, where two act as radical *S*-adenosylmethionine (AdoMet) enzymes (HydE and HydG) and the other as a GTPase (HydF). In this review, biological CO and CN<sup>−</sup> genesis mechanisms will be assessed with specific focus on [FeFe]-hydrogenase maturation.

## 1 Introduction

Hydrogenases represent a class of metalloenzymes that catalyze the reversible oxidation of hydrogen ( $2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2$ ).<sup>[1]</sup> Hydrogenases are generally classified by the metal content of their active sites, forming three classes termed [Fe]-, [FeFe]-, and [NiFe]-hydrogenases, and these classes are distinct in their metal contents, physiology and in their phylogenetic history.<sup>[2]</sup> The [Fe]-hydrogenases are functionally different from [NiFe]- and [FeFe]-hydrogenases and have only been found in a small group of methanogenic archaea.<sup>[2]</sup> The [Fe]-hydrogenase enzymes function as hydrogen forming methylenetetrahydromethanopterin dehydrogenases (Hmd).<sup>[3]</sup> The [Fe]-hydrogenase performs a different type of reaction from the other two types of hydrogenases in that it catalyzes the reversible reduction of N<sup>5</sup>,N<sup>10</sup>-methyltetrahydromethanopterin (CH–H<sub>4</sub>MPT<sup>+</sup>) with H<sub>2</sub> to N<sup>5</sup>,N<sup>10</sup>-methylenetetrahydromethanopterin (CH<sub>2</sub>=H<sub>4</sub>MPT) and a proton (CH–H<sub>4</sub>MPT<sup>+</sup> + H<sub>2</sub> ⇌ CH<sub>2</sub>=H<sub>4</sub>MPT + H<sup>+</sup>). The [FeFe]- and [NiFe]-hydrogenases are found in a more diverse set of organisms and have been studied in greater detail.<sup>[4–7]</sup> As of the writing of

this manuscript, [FeFe]-hydrogenases have been found within lower eukaryotes and bacteria; they have not been found associated with archaea or cyanobacteria. [NiFe]-hydrogenases are found only within bacteria, including cyanobacteria or archaea, but they have not been found within eukaryotes.<sup>[2,4]</sup>

The hydrogenase enzyme classes are evolutionary unrelated, yet similar structural traits are apparent among the enzyme classes.<sup>[4]</sup> All three hydrogenase classes contain a redox-inactive low-spin iron that is ligated by five or six ligands arranged as a distorted square pyramid or octahedron. These ligands include CO, CN<sup>−</sup>, or pyridinol, which are all  $\pi$ -accepting ligands, and these ligands are oriented *cis* to each other. Also a thiolate sulfur coordinates the iron *trans* to a diatomic molecule in all three classes of hydrogenase. Each of these low spin iron centers act together with a redox active substituent, which are methenyl-H<sub>4</sub>MPT<sup>+</sup> in the [Fe]-hydrogenase, the distal iron in the [FeFe]-hydrogenase, and nickel in the [NiFe]-hydrogenase; the position of this redox active center is similar in each hydrogenase class.<sup>[8,9]</sup> CO and CN<sup>−</sup> active site ligands support hydrogen activation, and it appears that each class of hydrogenase has evolved different approaches for incorporating these essential ligands.<sup>[10]</sup> Recent findings have shown that the [FeFe]-hydrogenase maturase HydG can catalyze the formation of both CN<sup>−</sup> and CO for incorporation into the H-cluster.<sup>[11,12]</sup> These results yield the first instance reported in a biological system of the simultaneous formation of both CN<sup>−</sup> and CO. This review examines other biological

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systems that synthesize  $\text{CN}^-$  and CO, assesses the recent findings of the  $\text{CN}^-$  and CO generating mechanism of the [FeFe]-hydrogenase maturase HydG, and discusses the implications these findings have on total [FeFe]-hydrogenase activation.

## 2 Biological Production and Roles of $\text{CN}^-$ and CO

### 2.1 Biological Cyanogenesis

Cyanide ( $\text{HCN}$  or  $\text{CN}^-$ ) genesis (cyanogenesis) mechanisms are prevalent throughout biology and are known to

occur in algae, bacteria, fungi, and plants.<sup>[13]</sup> Although the mechanisms employed are diverse, in each case, the nitrogen and carbon atoms of  $\text{CN}^-$  are derived from an amino acid precursor.<sup>[13]</sup> It has been postulated that  $\text{CN}^-$  can be generated in plants from non-amino acid derived glyoxylate oxime, although this has not been demonstrated experimentally.<sup>[14]</sup> In higher plants, the L-amino acids Val, Ile, Leu, Phe, Tyr, or the nonprotein amino acid cyclopentenyl glycine are oxidized to yield  $\text{CN}^-$ , glucose, and an aldehyde or ketone for chemical defense against herbivores and pathogens.<sup>[15]</sup>

This pathway of  $\text{CN}^-$  genesis has been fairly well elucidated in *Sorghum bicolor* L. Moench, where tyrosine is used



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John Peters was born in 1965. He received a B.S. in Microbiology from the University of Oklahoma and a Ph.D. from Virginia Tech. He was a NIH postdoctoral fellow at Cal Tech before joining Utah State University as Assistant Professor in 1997 where he was the recipient of the Camille Dreyfus Teacher-Scholar Award. In 2002, he joined Montana State University and is currently Professor of Chemistry and Biochemistry and Director of the NASA funded Astrobiology Biogeochemistry Research Center and the MSU Thermal Biology Institute. His research interests are in complex iron–sulfur enzymes, specifically hydrogenase and nitrogenase structure, function and biosynthesis.



Joan Broderick was born in 1965. She received a B.S. in chemistry from Washington State University and a Ph.D. from Northwestern University. She was an American Cancer Society postdoctoral fellow at MIT before joining the faculty at Amherst College as Assistant Professor in 1993. She moved to Michigan State University in 1998 and to Montana State University in 2005, where she is currently Professor of Chemistry and Biochemistry. Her research interests are in mechanistic bioinorganic chemistry, with a particular focus on enzymes utilizing iron–sulfur clusters to catalyze radical reactions.

as the substrate.<sup>[13,16]</sup> It has been shown that cyanogenesis requires the use of several reaction steps (Figure 1) mediated by five separate enzymes.<sup>[13,16]</sup> Two of these enzymes are from the P450 heme-containing enzyme superfamily, with the remaining three being a glycosyltransferase, a  $\beta$ -glucosidase, and a  $\alpha$ -hydroxynitrile lyase.<sup>[17,18]</sup> One of the P450-like enzymes catalyzes two *N*-hydroxylations of the amino group on the amino acid, where it dehydrates, decarboxylates, and isomerizes the substrate to a *Z*-aldoxime.<sup>[17,18]</sup> Then the other P450-like enzyme catalyzes the NADPH dependent dehydration and C-hydroxylation of the aldoxime to an  $\alpha$ -hydroxynitrile.<sup>[17]</sup> The  $\alpha$ -hydroxynitrile is then glycosylated by a uridine diphosphate (UDP) glucose dependent glycosyltransferase to form a cyanogenic glucoside.<sup>[19,20]</sup> Catabolism of the cyanogenic glucoside is initiated by enzymatic hydrolysis by a  $\beta$ -glucosidase to yield  $\alpha$ -hydroxynitrile and glucose, and the final dissociation is usually catalyzed by an  $\alpha$ -hydroxynitrile lyase generating HCN and a ketone or aldehyde.<sup>[21,22]</sup>

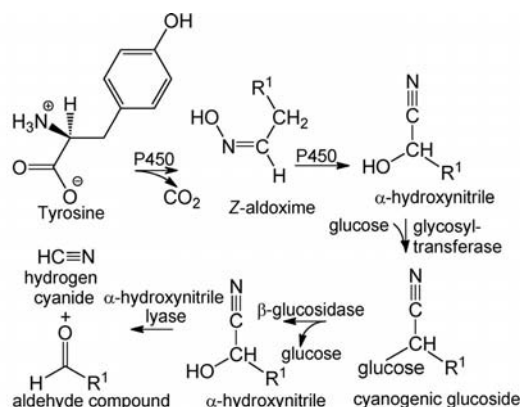


Figure 1. Schematic representation of the five step catalyzed cyanogenesis in *Sorghum bicolor* L. Moench. Tyrosine is first converted to a *Z*-aldoxime and carbon dioxide, and then to a  $\alpha$ -hydroxy nitrile by two separate P450-like enzymes. This is then followed by a glycosylation event by a glycosyltransferase; an elimination of the  $\beta$ -glycosyl group back to a  $\alpha$ -hydroxynitrile; and a final cleavage of the  $\alpha$ -carbon and  $\beta$ -carbon bond to form cyanide and an aldehyde compound.

Other well studied cases of cyanogenesis are in the bacteria, *Chromobacterium violaceum*, and in some but not all strains of *Pseudomonads*.<sup>[23]</sup> It is suggested that these organisms perform the oxidation of the amino acid glycine to HCN (Figure 2) in order to remove excess of this primary metabolite.<sup>[23]</sup> HCN and  $\text{CO}_2$  are produced in a 1:1 stoichiometric ratio by oxidative decarboxylation of glycine, which is thought to occur through a dehydroglycine intermediate or an oxime species analogous to the plant system.<sup>[23,24]</sup> The oxidation of glycine to HCN is catalyzed by a single enzyme, which is encoded by three structural genes *hcnABC* and is termed HCN-synthase. HCN-synthase is an oxygen-sensitive membrane bound protein in *C. violaceum* and in *Pseudomonas* and is thus difficult to purify. As a result, HCN-synthase has only been partially purified, and little is known about the mechanism by which this enzyme oxidizes glycine.<sup>[25,26]</sup> It is clear, however, that the reaction

occurs through a decarboxylation mechanism, since the C–N bond is conserved and the carboxy carbon is maintained on  $\text{CO}_2$  in the reaction products.<sup>[24,27]</sup>

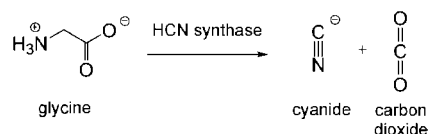


Figure 2. The cyanogenesis reaction that occurs in the bacterium *Chromobacterium violaceum* and in some but not all strains of *Pseudomonads*. Glycine acts as the substrate and is decarboxylated to yield cyanide and carbon dioxide.

## 2.2 Carbon Monoxide Biosynthesis

Carbon monoxide is produced from the incomplete combustion of organic materials that are produced naturally by bacteria, plants, and animals.<sup>[28]</sup> Biological CO production and metabolism is significant to the global carbon cycle and has been reviewed exhaustively elsewhere.<sup>[29–31]</sup> The primary enzymatic pathways that generate CO include the Wood–Ljungdahl pathway and the carbon monoxide dehydrogenase pathway; in the latter CO has been observed to be an obligatory intermediate.<sup>[30,32]</sup> CO is also produced during several other biological processes, including heme degradation by heme oxygenase, and conversion of *S*-methylthioadenosine to methionine, as well as from amino acid metabolism in bacteria.<sup>[33,34]</sup> Some organisms survive solely on CO, acting as both a carbon and energy source.<sup>[35]</sup> CO is a source of low potential electrons with a midpoint potential at pH 7 equaling  $-528$  mV, where it is at the same redox state as a carbonyl group in acetyl-CoA.<sup>[36]</sup> However, CO is also toxic, such that tight coupling of CO production and utilization is necessary to minimize toxic effects.<sup>[28]</sup>

## 2.3 Cyanide and Carbon Monoxide as Inorganic Ligands

The CO and  $\text{CN}^-$  ligands that are bound to the active site metal centers of the hydrogenases are important in tuning chemical reactivity of the coordinated iron or nickel.<sup>[37]</sup> Both heterodiatomic ligands are strong  $\pi$ -acids with vacant  $\pi^*$  orbitals that allow for substantial back-bonding with low-valent metal centers such as the Fe in hydrogenase active sites.<sup>[38]</sup> When bound to a low-valent metal, these ligands accept electron density from a filled metal *d* orbital into an empty antibonding orbital of the C–N or C–O bond.<sup>[38,39]</sup> While the negative charge of the  $\text{CN}^-$  ion decreases its  $\pi$ -bonding ability relative to CO, hydrogen bonding mitigates the negative charge effect of  $\text{CN}^-$  and increases its  $\pi$ -bonding.<sup>[38]</sup> Computational work by Stiebritz and Reiher have shown that the iron centers of hydrogenases exhibit general reactivity principles related to the presence of CO and  $\text{CN}^-$  in the first coordination sphere.<sup>[40]</sup>



### 3 Hydrogenase Enzymes

#### 3.1 Hydrogenase Structures

##### 3.1.1 [Fe]-Hydrogenase Structure

The Fe only hydrogenase (also termed Hmd or iron–sulfur cluster free hydrogenase) was originally thought to be devoid of metal cofactors.<sup>[41]</sup> It has now been determined through spectroscopic studies as well as X-ray crystallography that a single iron is coordinated to unique nonprotein ligands in its active site (Figure 3).<sup>[8]</sup> The [Fe]-hydrogenase is a dimer composed of two identical subunits of molecular mass 38 kDa and contains two catalytically essential iron centers per dimer.<sup>[42,43]</sup> Mössbauer spectra has shown that the cofactor contains a mononuclear iron centre and that the electronic state of the iron site is low spin  $\text{Fe}^0$  or low spin  $\text{Fe}^{\text{II}}$ .<sup>[44]</sup> Fourier transform infrared (FTIR) spectroscopy revealed two CO ligands that are bound to the iron site at  $90^\circ$  angles, and an additional CO or cyanide ligand was found to be coordinated to the iron site when CO or KCN was added to Hmd.<sup>[45,46]</sup> From X-ray absorption data, it was inferred that the Fe was most likely coordinated by two CO ligands, one sulfur, and one or two N or O ligands.<sup>[47]</sup>

The crystal structure of the apo-Hmd hydrogenase from *Methanocaldococcus jannaschii* has revealed an enzyme composed of one central and two similar peripheral domains.<sup>[48]</sup> The binding site for the iron cofactor is located in a cleft between the central and peripheral domains on a cysteine, which has been shown to be required for enzyme activity.<sup>[48]</sup> The crystal structure of the *Methanocaldococcus jannaschii* [Fe]-hydrogenase holoenzyme has also been determined, and it displays a unique active site containing an iron center bound by two *cis*-CO ligands, the pyridinol nitrogen, a thiolate ligand, a acyl carbon from the guanylyl pyridinol cofactor, and a vacant site where  $\text{H}_2$  possibly

binds.<sup>[8,49,50]</sup> The geometry of the ligand environment is distorted square pyramidal or octahedral, depending on the occupancy of the ligand at the sixth site.<sup>[8,49,50]</sup>

##### 3.1.2 [NiFe]-Hydrogenase Structure

[NiFe]-hydrogenases contain a heterobimetallic Ni and Fe active site, and these enzymes generally are heterodimers of a large (60 kDa) and a small (30 kDa) subunit containing several accessory iron–sulfur clusters.<sup>[10]</sup> The first [NiFe]-hydrogenase crystal structure was determined from *Desulfovibrio gigas*, which revealed the presence of Ni between the  $\alpha$  and  $\beta$  domains of the large subunit of the hydrogenase.<sup>[51]</sup> A later structure appeared to have electron density belonging to diatomic ligands bound to a heterobimetallic Ni and Fe active site.<sup>[51,52]</sup> Subsequent high resolution structures have confirmed this finding and have helped to determine the identity of the diatomic ligands, as well as elucidating other structural features such as a hydrophobic gas channel, which likely conducts hydrogen to the active site.<sup>[10,53]</sup> FTIR spectroscopic studies have revealed the presence of one CO and two  $\text{CN}^-$  ligands on the active site, and it also yielded results indicating a stoichiometry of  $\text{NiFe}(\text{CN})_2(\text{CO})$  (Figure 3).<sup>[54]</sup>

[Fe]- and [FeFe]-hydrogenases are purified or activated under anaerobic conditions, whereas some [NiFe]-hydrogenases are oxygen tolerant and can be purified aerobically.<sup>[48,55–58]</sup> FTIR and electron paramagnetic resonance (EPR) spectroscopic studies on the NAD-reducing hydrogenase from *Cupriavidus necator* suggested this tolerance may be due to the presence of two additional cyanides.<sup>[59]</sup> One of the cyanides was proposed to be bound to the Ni atom and the other to the Fe, providing an active site composition of  $\text{Ni}(\text{CN})\text{Fe}(\text{CN})_3(\text{CO})$ .<sup>[59]</sup> Deletion of the gene encoding for the auxiliary protein HypX was shown to eliminate oxygen tolerance. The source of the extra cyanides in this case is unknown.<sup>[60]</sup>

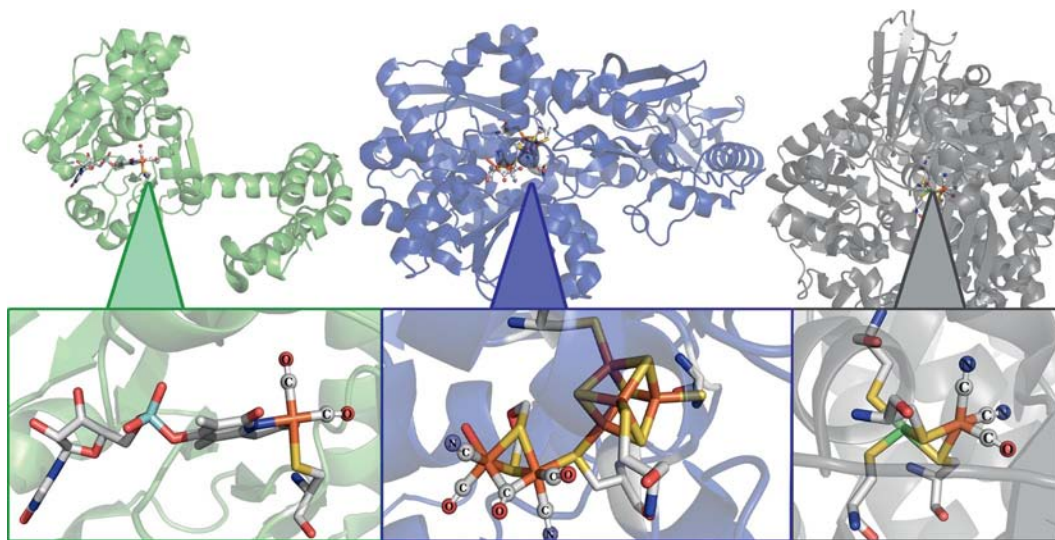


Figure 3. Crystal structures of the [Fe]- (printed in green) 3F47, [FeFe]- (blue) 3C8Y, and [NiFe]-hydrogenase (grey) 1YRQ, and close-ups of each individual enzyme active site. Atoms labeled as: carbon (grey), oxygen (red), nitrogen (blue), sulfur (yellow), phosphorus (cyan), iron (rust), and nickel (green). Carbon monoxide and cyanide groups are represented as ball and stick representations.

### 3.1.3 [FeFe]-Hydrogenase Structure

[FeFe]-hydrogenases can exist as either monomers or heteromers, and they typically contain a varying number of accessory iron–sulfur clusters (F-clusters) that shuttle electrons to or from the active site H-cluster buried deep within the enzyme.<sup>[61]</sup> The crystal structure of the [FeFe]-hydrogenase from the Gram-positive anaerobe *Clostridium pasteurianum* first revealed the unusual H-cluster,<sup>[62,63]</sup> and FTIR spectroscopy allowed for the assignment of the coordination of CO and CN<sup>−</sup> ligands.<sup>[64,65]</sup> The H-cluster was shown to comprise a [4Fe-4S] cubane bridged by a cysteine to a [2Fe] subcluster. The proximal iron of the [2Fe] subcluster is coordinated by one CN<sup>−</sup> ligand and one CO ligand and is bridged by one carbon monoxide and a dithiolate ligand of undetermined composition to the distal Fe. The distal Fe is further coordinated by one CO ligand and one CN<sup>−</sup> ligand in the oxidized state (Figure 3).<sup>[61,63,64]</sup> The composition of the dithiolate bridge has not been determined by direct chemical methods. It was initially proposed that the dithiolate ligand could be a dithiopropyl ligand.<sup>[61]</sup> Crystallographic refinement studies have been inconclusive in determining the composition of the dithiolate bridge, and they have suggested the dithiolate ligand being a dithiomethyl-ether, dithiopropyl, or dithiomethylamine.<sup>[66–68]</sup> Recent results from hyperfine sublevel correlation spectroscopy (HYSCORE) and density functional theory (DFT) calculations indicate that the dithiolate bridge is dithiomethylamine.<sup>[69]</sup>

## 3.2 Biosynthesis of Hydrogenase Active Site Clusters

### 3.2.1 [Fe]-Hydrogenase Biosynthesis

Maturation of the active site of the Hmd hydrogenase is less well understood than that of either the [NiFe]- or [FeFe]-hydrogenases. A radical-AdoMet enzyme appears to be involved in the assembly process, and by analogy to HydG in [FeFe]-hydrogenase biosynthesis (vide infra), could be involved in CO ligand synthesis or insertion during the maturation of the [Fe]-hydrogenase.<sup>[70]</sup> Further, Turrell et al. hypothesize, based on insight gained from the synthesis of an [Fe]-hydrogenase active site mimic, that the Fe(CO)<sub>2</sub>S is formed initially and is followed by the amidation of the iron to the guanylyl pyridinol cofactor.<sup>[71]</sup> A full mechanistic understanding for the biosynthesis of this cofactor, however, is currently outstanding.

### 3.2.2 [NiFe]-Hydrogenase Biosynthesis

In 2001, Paschos et al. revealed carbamoyl phosphate as a substrate for [NiFe]-hydrogenase maturation.<sup>[72]</sup> Carbamoyl phosphate is synthesized from ammonia and hydrogen carbonate by carbamoyl phosphate synthase; under nitrogen-limited conditions, the ammonia is derived from glutamine or glutamate by either glutamate dehydrogenase or glutaminase aminohydrolase.<sup>[73–75]</sup> It was shown that *Escherichia coli* lacking a *carB* gene (encoding the catalytic portion of carbamoyl phosphate synthase) displayed no hy-

drogenase activity.<sup>[72]</sup> Further, no proteolytic processing was observed for the large subunit of the [NiFe]-hydrogenase, suggesting incomplete maturation.<sup>[72]</sup>

It was hypothesized that carbamoyl phosphate could be the substrate to produce either CN<sup>−</sup> and/or CO ligands, and it was later determined that CN<sup>−</sup> generation within [NiFe]-hydrogenases occurs by maturases HypE and HypF.<sup>[76]</sup> Initial results showed that HypF hydrolyzes ATP and adenylates carbamoylphosphate; Reissmann et al. in 2003 showed that cyanogenesis could occur from this carbamoyladenylate via the HypF and HypE maturases.<sup>[77]</sup> They found in this system cyanogenesis occurs in a highly controlled fashion, and CN<sup>−</sup> was not freely liberated from the enzyme under reaction conditions unless in the presence of an iron analogue thought to be similar to the inorganic Fe found in the HypC/HypD complex.<sup>[76]</sup> They proposed a mechanism for the biosynthesis of CN<sup>−</sup> as shown in Figure 4.<sup>[76]</sup> The carbamoyltransferase HypF hydrolyzes ATP and transfers the carboxamido group of carbamoylphosphate to a C-terminal cysteine in the conserved PRIC motif found in HypE. This modified HypE then transforms the thiocarbamide to thiocyanate by hydrolyzing ATP and dehydrating the thiocarbamide group. After the HypE thiocyanate transformation, HypE can transfer its cyano group to either an electrophilic or nucleophilic iron, which they proposed either state could be bound to the HypC/HypD complex.<sup>[76]</sup> In 2007, Lenz et al. further demonstrated that CO and not CN<sup>−</sup> are derived from carbamoyl phosphate. Using a heterologous expression system of *C. necator* H16 [NiFe]-hydrogenase in *E. coli* with *carAB* and *argG* gene deletions, it allowed the use of FTIR analysis to assign peaks from cyanide. This was due to a shift of the cyanide peaks to a lower frequency as a result of expressions in minimal media supplemented with L-[ureido-<sup>13</sup>C] citrulline, and this allowed recovery of carbamoyl phosphate production and selective labeling of CN<sup>−</sup> on the hydrogenase.<sup>[78]</sup>

The remaining aspects of maturation of the [NiFe]-hydrogenase has been reviewed extensively elsewhere.<sup>[79–81]</sup> In short, the maturation enzyme HypC is known to complex with the apo-large subunit of the [NiFe]-hydrogenase and is believed to act as a chaperone. HypD, which contains a [4Fe-4S] cluster, also forms a complex with HypC, and this protein is implicated in transferring cluster ligands to the active site. HypF and HypE create the CN<sup>−</sup> ligands, while the origin of CO remains undetermined. The mechanism behind the insertion of the catalytic cluster is still hypothetical, but it appears that the CO and CN<sup>−</sup> ligands are first bound to the [4Fe-4S] cluster on HypD before being inserted into the iron scaffolded on HypC. In addition, it is thought that the cluster is composed of Fe(CN)<sub>2</sub>CO, which is carried by HypC onto the large subunit. After iron insertion, HypB and HypA act in concert to insert Ni into the active site. While HypA is the delivery agent for inserting nickel into the active site, HypB appears to allosterically regulate HypA, and only after hydrolysis of GTP, HypB induces HypA to insert the metal. At the end of maturation in *E. coli*, an endopeptidase cleaves the C-terminus of the

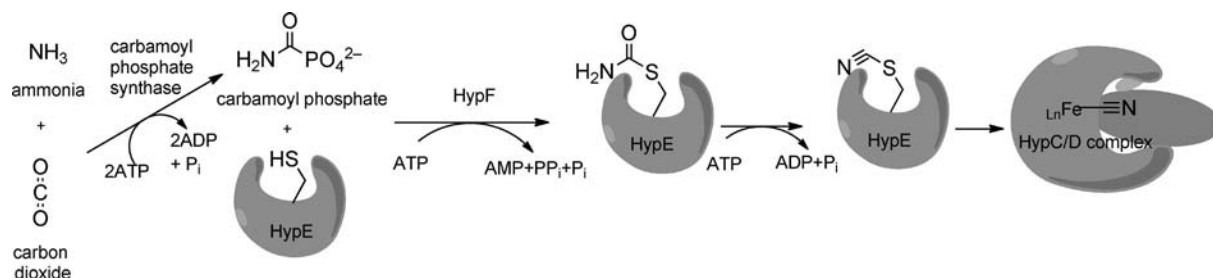


Figure 4. Schematic of cyanogenesis occurring during the maturation of a [NiFe]-hydrogenase. Carbamoyl phosphate is synthesized from hydrogen carbonate and  $\text{NH}_3$  in an ATP-dependent reaction. HypF hydrolyzes ATP and transfers the carboxamido group of carbamoyl-phosphate to a C-terminal cysteine in the conserved PRIC motif found in HypE. This modified HypE then transforms the thiocarbox-amide to thiocyanate in an ATP-dependent reaction. After the HypE thiocyanate transformation, HypE transfers its cyano group to the HypC/HypD complex.

large subunit, and the active site becomes encapsulated within the shell of the large subunit. The portion of the hydrogenase that is cleaved is highly conserved, and it appears that uncleaved C-terminus keeps the active site fold accessible for maturation.

### 3.2.3 [FeFe]-Hydrogenase Biosynthesis

The genes necessary to produce an active [FeFe]-hydrogenase were identified by random insertional mutagenesis in *Chlamydomonas reinhardtii*; knocking out three specific genes (other than the gene encoding the hydrogenase itself) resulted in the complete lack of hydrogenase activity.<sup>[82]</sup> The three genes included two encoding radical *S*-adenosyl-methionine (AdoMet) proteins (termed HydE and HydG), and one encoding a GTPase (HydF).<sup>[83]</sup> Further characterization of HydE and HydG showed that both enzymes bind multiple iron–sulfur clusters and reductively cleave AdoMet.<sup>[84]</sup> HydF from *Thermatoga maritima* was also characterized and shown to be an iron–sulfur binding GTPase.<sup>[85]</sup> By analogy to HypB (*vide supra*), it was speculated that HydF might be involved in a metal insertion reaction.<sup>[85]</sup>

Utilization of an *E. coli* heterologous expression system with different [FeFe]-hydrogenase genetic backgrounds has provided critical insight into the process of its maturation, and it has also supported the idea of scaffold-based synthesis of the 2Fe subcluster on HydF.<sup>[86,87]</sup> Inactive  $\text{HydA}^{\Delta\text{EFG}}$  (the hydrogenase structural protein expressed in the absence of accessory proteins) was shown to be activated *in vitro* by addition of *E. coli* extracts of heterologously expressed HydE, HydF, and HydG, and this raised the question of the minimum requirement of  $\text{HydA}^{\Delta\text{EFG}}$  activation.<sup>[86]</sup> It was subsequently found that  $\text{HydF}^{\text{EG}}$  (HydF expressed in the presence of HydE and HydG but then purified away from these two maturases) alone was capable of *in vitro* activation of  $\text{HydA}^{\Delta\text{EFG}}$ ; thus HydF appeared to act as a scaffold delivering the activating component to  $\text{HydA}^{\Delta\text{EFG}}$ .<sup>[86]</sup> The stepwise nature of this activation process reveals the ability of HydG and HydE to synthesize the activating element on HydF for later activation of HydA.<sup>[87]</sup> A recent crystal structure of the heterologously expressed *Chlamydomonas reinhardtii*  $\text{HydA}^{\Delta\text{EFG}}$  apo-hydrogenase has revealed the presence of a [4Fe-4S] cluster, which was presumably inserted by general Fe-S synthetic

host machinery.<sup>[88]</sup> It is hypothesized that a CO,  $\text{CN}^-$ , and dithiolate-decorated [2Fe] subcluster is inserted by HydF into HydA, resulting in a conformational change of the HydA protein to close the active site pocket and complete the maturation process.<sup>[88]</sup> Furthermore, it has been demonstrated that delivery of the decorated [2Fe] subcluster to HydA is not GTPase driven, but rather it is thought that GTPase activity is involved in synthesis of the 2Fe subcluster precursor.<sup>[89]</sup>

Spectroscopic characterization of HydF expressed in the presence and absence of maturases HydG and HydE ( $\text{HydF}^{\text{EG}}$  and  $\text{HydF}^{\Delta\text{EG}}$ , respectively) has also provided evidence that HydF functions as a scaffold.<sup>[12,87,89]</sup> The UV/Vis difference spectrum ( $\text{HydF}^{\Delta\text{EG}} - \text{HydF}^{\text{EG}}$ ) shows features characteristic of a [2Fe-2S] cluster, suggesting the “loss” of such a cluster on HydF in the presence of HydE and HydG.<sup>[87]</sup> Further, EPR spectroscopy has revealed that HydF contains a [2Fe-2S] and a [4Fe-4S] signal in the genetic absence of HydE and HydG, but only the [4Fe-4S] signal when expressed together with HydE and HydG.<sup>[89]</sup> FTIR spectroscopy provided insight into the fate of the “lost” [2Fe-2S] cluster by providing evidence for the presence of  $\text{CN}^-$  and CO ligands in HydF.<sup>[89,90]</sup> FTIR data has been obtained for HydF isolated from the host organism *Clostridium acetobutylicum* and heterologously expressed  $\text{HydF}^{\text{EG}}$  in *E. coli*.<sup>[89,90]</sup> These results suggest that the enzymatic activities of HydE and HydG serve to convert a standard [2Fe-2S] cluster on HydF into a CO and  $\text{CN}^-$  ligated [2Fe] H-cluster precursor.

In 2006, we analyzed several key characteristics of the [FeFe]-hydrogenase maturation system to develop three fundamental hypotheses in the hopes of advancing the field of [FeFe]-hydrogenase research and to guide our own research.<sup>[91]</sup> The first hypothesis resulted from the observation that the maturase genes were found in an array of organisms and a variety of environmental niches, in many cases associated with specialized and constrained metabolisms. It was, therefore, postulated that 1) since the only commonality among these organisms was central metabolism, then the common substrates for H-cluster biosynthesis must be a central metabolite such as an amino acid. The second hypothesis resulted from the observation that [2Fe-2S] clusters have highly reactive sulfides, and that alkylation of



these sulfides would shift reactivity to the irons. Thus, it was proposed that 2) synthesis of the dithiolate ligand by insertion of bridging sulfides into C–H bonds would be an early step in synthesis of the 2Fe subcluster. The final hypothesis came from the idea that CO and  $\text{CN}^-$  should be generated in a simple and controlled fashion since they are metabolic poisons. It was proposed that 3) CO and  $\text{CN}^-$  would be generated from one central metabolic source such as an amino acid, with the simplest one being glycine, by controlled decomposition on a metal cluster.<sup>[91]</sup>

## 4 Radical *S*-Adenosylmethionine Enzymes

### 4.1 Radical *S*-Adenosylmethionine Enzyme Overview

Radical *S*-Adenosylmethionine (AdoMet) enzymes comprise a superfamily of proteins that catalyze a diverse series of chemical transformations through radical mediated mechanisms.<sup>[92]</sup> Known reactions in this superfamily include sulfur insertion, organic cofactor biosynthesis, amino group rearrangements, DNA repair, glycy radical formation, and complex metal cofactor biosynthesis.<sup>[93,94]</sup> Radical AdoMet enzyme reactions are initiated by homolytic bond cleavage of *S*-adenosylmethionine S–C5' bond after reduction by a reduced  $[4\text{Fe-4S}]^{1+}$  cluster occupying the conserved  $\text{CX}_3\text{CX}_2\text{C}$  iron–sulfur binding motif. This initial step yields a 5' deoxyadenosyl radical (dAdo $\cdot$ ) (Figure 5).<sup>[94]</sup> The dAdo $\cdot$  then abstracts a hydrogen atom to generate a substrate radical and dAdoH. AdoMet most commonly acts as a substrate and thus is consumed during turnover; in some cases, however, a product radical reabstracts  $\text{H}^\cdot$  from dAdoH to regenerate dAdo $\cdot$ , making the AdoMet cleavage reversible. In the latter mechanism, AdoMet acts as a cofactor rather than a substrate.<sup>[94,95]</sup>

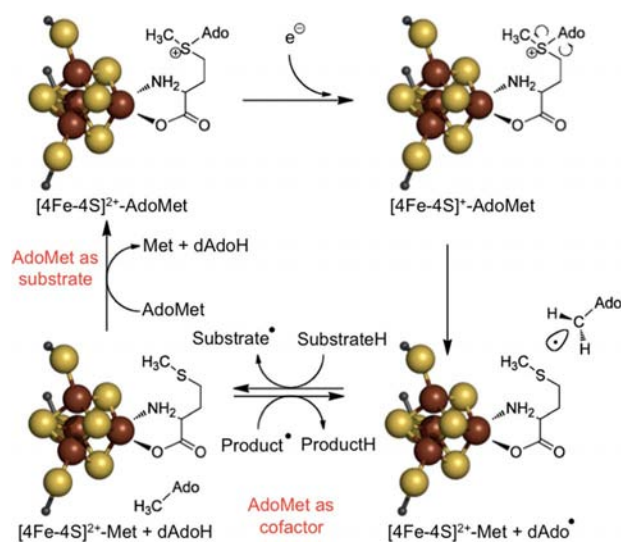


Figure 5. Common mechanistic steps for reactions catalyzed by radical AdoMet enzymes. The iron–sulfur cluster was generated in PyMol from the structure 3CB8.pdb.

### 4.2 Preliminary Characterization of Radical AdoMet Enzymes HydE and HydG

Radical AdoMet enzymes employ iron–sulfur clusters as a means for radical production.<sup>[94]</sup> HydE and HydG both contain two iron–sulfur cluster binding motifs, where one is the canonical N-terminal  $\text{CX}_3\text{CX}_2\text{C}$  radical AdoMet motif.<sup>[83,93]</sup> The second iron–sulfur cluster binding motif in both proteins is near the C-terminus, with HydG having a strictly conserved  $\text{CX}_2\text{CX}_{22}\text{C}$  motif and HydE having a somewhat less conserved  $\text{CX}_7\text{CX}_2\text{C}$  motif.<sup>[83,96]</sup> EPR and UV/Vis spectroscopic characterization of HydE and HydG from *Thermatoga maritima* has indicated the presence of at least one  $[4\text{Fe-4S}]^{1+}$  cluster in each of the enzymes, as well as a mixture of  $[4\text{Fe-4S}]$  and  $[2\text{Fe-2S}]$  clusters, and this was gauged by microwave power saturation and temperature dependent properties of the clusters.<sup>[84]</sup> However, the EPR signals were too complex to definitively identify the cluster composition. This ambiguity is likely due to the substoichiometric quantities of iron consistently present in the enzyme samples.<sup>[84]</sup> Furthermore, it has been shown that the strictly conserved iron–sulfur binding domains in HydE and HydG are essential for HydA activation.<sup>[82]</sup> Single point mutations of the cysteine residues of the radical AdoMet domain of HydE and HydG or the accessory Fe–S domain of HydG were shown to greatly impair HydA activation.<sup>[82]</sup> It has also been shown that point mutations in the accessory Fe–S domain of HydE have no impact on overall HydA activation.<sup>[97]</sup>

### 4.3 Radical-Initiated Amino Acid Decomposition by ThiH and HydG

Several radical AdoMet enzymes are known to act on amino acid substrates, including lysine-2,3-aminomutase (LAM), tyrosine lyase (ThiH), and the glycy radical activating enzymes that generate catalytically essential radicals in enzymes such as the anaerobic ribonucleotide reductase (ARR), pyruvate-formate lyase (PFL) and *p*-hydroxyphenylacetic acid decarboxylase.<sup>[92,94]</sup> HydG exhibits 27% sequence homology to ThiH, and both enzymes display similar patches of strictly conserved amino acids at the internal faces of the  $\beta$ -strands that define the barrel, suggesting that similar chemistry may take place in this conserved region of the two enzymes.<sup>[98]</sup>

ThiH catalyzes the radical cleavage of tyrosine via putative phenolic H atom abstraction to produce *p*-cresol and dehydroglycine, with the later being an intermediate in thiamine biosynthesis.<sup>[98–103]</sup> Two possible mechanisms for the radical AdoMet-mediated cleavage of tyrosine to dehydroglycine is shown in Figure 6.<sup>[101]</sup> A heterolytic  $\text{C}_\alpha\text{--C}_\beta$  bond cleavage would result in the formation of dehydroglycine and the *p*-cresolate radical anion.<sup>[101]</sup> Alternatively, a homolytic  $\text{C}_\alpha\text{--C}_\beta$  bond cleavage would yield a substrate glycy radical and a *p*-quinone methide intermediate, where oxidation of the radical via one-electron removal would also potentially yield dehydroglycine and a resonance-stabilized *p*-cresolate radical anion. Reduction of the methide

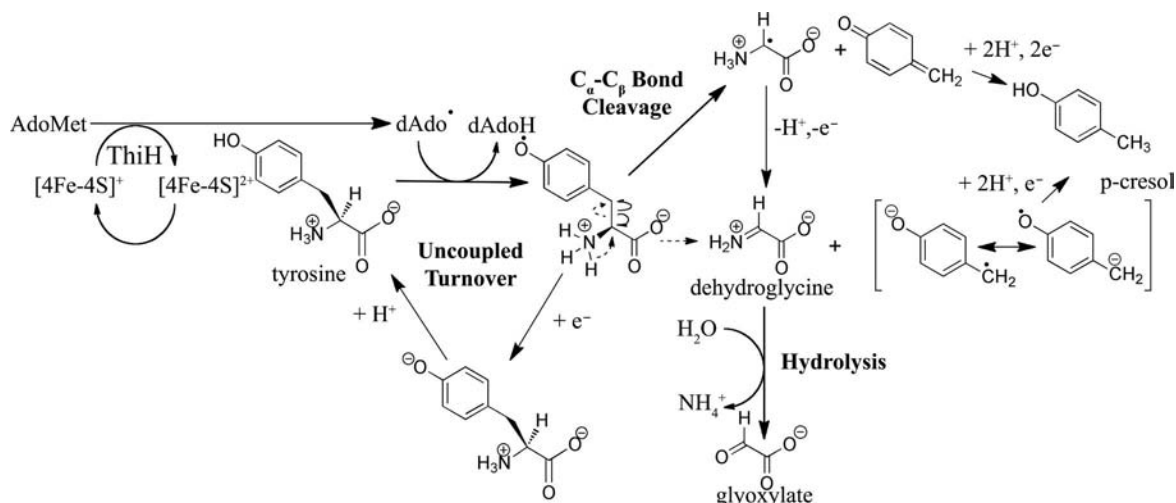


Figure 6. Proposed mechanism for tyrosine decomposition for the radical AdoMet tyrosine lyase ThiH.

results in formation of *p*-cresol.<sup>[101]</sup> Dehydroglycine (2-iminoacetic acid) has been proposed as the primary reaction intermediate, since glyoxylate (the hydrolyzed product of dehydroglycine) is produced equimolar to *p*-cresol upon absence of the thiazole synthase ThiG, the sulfur donor ThiFS thiocarboxylate, or 1-deoxyxylulose 5-phosphate (Dxp).<sup>[99,100,104,105]</sup> Therefore, ThiH is thought to require ThiG, ThiFS, and Dxp to form a complex to stabilize the proposed intermediate dehydroglycine and incorporate it into Dxp to form the thiazole moiety of thiamin phosphate.<sup>[100,101]</sup> Consistent with the sequence similarity to ThiH, initial results by Pilet et al. showed that HydG could cleave tyrosine and form *p*-cresol as a product.<sup>[98]</sup> These results suggested a similar mechanism of tyrosine cleavage in these two enzymes, and Pilet hypothesized that a dehydroglycine intermediate could also be formed in the HydG-

catalyzed reaction.<sup>[98]</sup> This hypothesis is supported by the idea that it is challenging to access an oxidized glycine intermediate in the absence of O<sub>2</sub>, and therefore, tyrosine decomposition to dehydroglycine would serve an alternative means of oxidizing glycine through a radical-mediated mechanism.<sup>[106]</sup>

## 5 Cyanogenesis by the [FeFe]-Hydrogenase Maturase HydG

Identification of tyrosine as the substrate for HydG was an important step forward in elucidating the mechanism of H-cluster biosynthesis; however, it did not answer the question as to which part of the H-cluster was derived from the tyrosine cleaved by HydG.<sup>[98]</sup> We, together with our collabo-

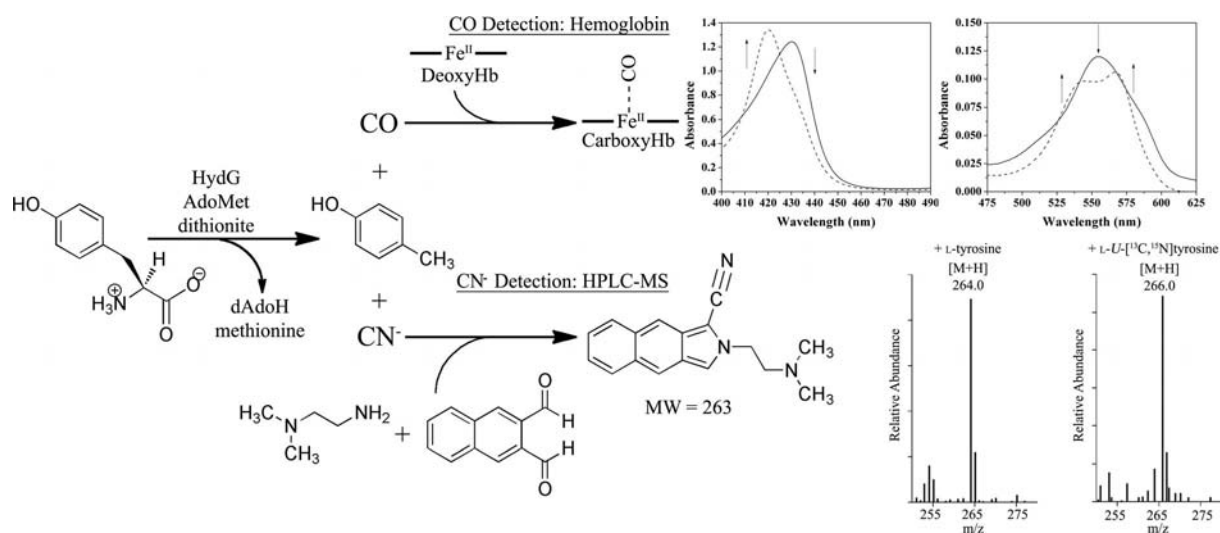


Figure 7. Characterization of cyanide and carbon monoxide as products of HydG catalyzed tyrosine cleavage. Produced cyanide was detected by HPLC and LC-MS after derivatization with a fluorescent 1-cyanobenz[1]isoindole. At specified time points, reaction aliquots were derivatized as shown. Carbon monoxide was detected by including deoxyhemoglobin (deoxyHb) in the HydG assay, and then utilizing UV/Vis spectroscopy to observe the time-dependent conversion of deoxyHb to carboxyhemoglobin (HbCO). UV/Vis spectra display HydG with dithionite, deoxyHb, and tyrosine in the absence (solid line) and presence (dashed line) of AdoMet.



rator Peter Roach, have addressed this question by carrying out assays for the production of CO and  $\text{CN}^-$  during HydG-catalyzed tyrosine cleavage. Our results show that HydG-catalyzed tyrosine cleavage results in the production of equimolar quantities of  $\text{CN}^-$  and *p*-cresol.<sup>[11]</sup> A slight excess of 5'-deoxyadenosine (dAdo) was also produced (1.3:1 dAdo to *p*-cresol), indicating a small degree of uncoupled AdoMet cleavage, as was also observed for ThiH.<sup>[94,101]</sup>  $\text{CN}^-$  was detected by derivatization with naphthalene-2,3-dicarbaldehyde to a fluorescent 1-cyanobenz[*f*]isoindole compound that allowed detection and quantification (Figure 7) by high performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS).<sup>[11]</sup> When a uniformly-labelled  $^{13}\text{C}$ ,  $^{15}\text{N}$ -tyrosine was used as a substrate, the resulting mass spectrum of the fluorescent complex increased by two mass units relative to the unlabelled complex, indicating tyrosine as the source of the  $\text{CN}^-$  ligand.<sup>[11]</sup>

The results suggest that HydG carries out chemistry similar to that as shown in Figure 6, but with the additional decomposition of dehydroglycine to  $\text{CN}^-$  (Figure 7). Consistent with this proposal, only small amounts of glyoxylate (< 10% of the quantity of *p*-cresol) were detected in the HydG assays.<sup>[11]</sup> In contrast, an equimolar concentration of glyoxylate to *p*-cresol was observed with the ThiH assays (performed in the absence of ThiG, ThiFS thiocarboxylate, and Dxp).<sup>[101]</sup>

## 6 CO Generation by the [FeFe]-Hydrogenase Maturase HydG

Given the working model that the primary intermediate in the decomposition mechanism is dehydroglycine, and that the C-2 carbon is the source of the  $\text{CN}^-$ , loss of the C-1 carboxylate fragment would yield a small molecule such as  $\text{CO}_2$  or CO.<sup>[11]</sup> It is interesting to note that amino acid oxidases produce  $\text{CN}^-$  and  $\text{CO}_2$  as byproducts, using amino acids as substrates (see Figures 1 and 2), and it has been proposed that the intermediate for the above mentioned amino acid oxidases involve dehydroglycine, where oxidative decarboxylation of dehydroglycine would yield  $\text{CO}_2$ .<sup>[107]</sup>

In order to examine the fate of the “carboxylate fragment” upon cleavage of tyrosine to produce *p*-cresol and  $\text{CN}^-$ , we developed an assay to detect CO produced during HydG turnover.<sup>[12]</sup> The assay involved inclusion of deoxyhemoglobin (deoxyHb) in addition to the typical components of a HydG assay; the high affinity of deoxyHb for CO, together with the characteristic spectral changes upon binding CO to Hb, provided a sensitive means of detecting CO produced during turnover.<sup>[12]</sup> Spectral changes in the Soret (a shift in the  $\lambda_{\text{max}}$  from 430 to 419 nm) and visible bands (splitting of the band at 555 nm to 540 and 569 nm) consistent with CO binding to hemoglobin were observed during HydG-catalyzed turnover of tyrosine (Figure 7).<sup>[12,108]</sup> Using uniformly  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled tyrosine together with FTIR spectroscopy of the reaction products, we showed that the CO bound to deoxyhemoglobin originated from tyrosine.<sup>[12]</sup>

CO production was found to be completely dependent on reconstitution of HydG, even though HydG as-isolated contained iron–sulfur clusters.<sup>[12]</sup> Spectroscopic evidence to date suggests that reconstitution introduces two discrete iron–sulfur clusters, consistent with the presence of two iron–sulfur cluster motifs in the HydG sequence. Reduced, reconstituted HydG displays a single fast relaxing signal ( $g = 2.03, 1.92, 1.90$ ) characteristic of  $[\text{4Fe-4S}]^{1+}$  clusters.<sup>[12]</sup> Addition of AdoMet to the reduced reconstituted enzyme splits this signal into two distinct rhombic signals ( $g_{\text{cluster1}} = 2.02, 1.93, 1.91$  and  $g_{\text{cluster2}} = 2.00, 1.87, 1.83$ ), presumably arising from two different iron–sulfur clusters on HydG. Both signals had a temperature dependence characteristic of  $[\text{4Fe-4S}]^{1+}$  clusters. In comparison, the reduced, as-purified HydG has an EPR signal characteristic of both  $[\text{4Fe-4S}]$  and  $[\text{2Fe-2S}]$  clusters.<sup>[12]</sup> The dependence of HydG-catalyzed production of CO on reconstitution suggests that both the radical AdoMet iron–sulfur cluster and a second iron–sulfur cluster play key roles in this fascinating chemical transformation.

## 7 Implications of HydG-Mediated CO/ $\text{CN}^-$ Synthesis

### 7.1 The Second Iron–Sulfur Clusters in HydG and HydE

Several radical AdoMet enzymes contain a second iron–sulfur cluster in addition to the radical AdoMet cluster, including MoaA, BioB, LipA, and HydE and HydG.<sup>[12,97,109–111]</sup> Evidence suggests that the second cluster in BioB and LipA serves as the source of sulfur for the sulfur insertion reaction these enzymes catalyze. In the case of MoaA, the second cluster is coordinated by the GTP substrate.<sup>[112]</sup> For the Hyd radical AdoMet enzymes, the role of the second cluster is unclear. It has been speculated that the second cluster in HydE, which can be either a  $[\text{2Fe-2S}]$  or a  $[\text{4Fe-4S}]$  cluster depending on the reconstitution conditions, may simply be an artefact of reconstitution and not functionally relevant.<sup>[97]</sup> However, our results for HydG suggest that the second iron–sulfur cluster is absolutely essential for CO formation.<sup>[12]</sup> Possible functional roles for the second cluster in HydG, which appears to be site-differentiated due to the presence of only three conserved cysteines in the cluster-binding motif, include the binding of dehydroglycine and/or binding of CO and  $\text{CN}^-$  prior to their transfer to the HydF scaffold. Lewis acid-promoted decarbonylation of amino acids is preceded in the literature, suggesting that similar chemistry could occur upon interaction of dehydroglycine with the second cluster of HydG.<sup>[113,114]</sup>

The HydG-mediated  $\text{CN}^-$  and CO biosynthesis from tyrosine implicates HydE in the formation of the bridging dithiolate of the 2Fe subunit. The substrate for HydE is currently unknown, however the synthesis of this dithiolate ligand has been proposed to involve chemistry analogous to that catalyzed by the radical AdoMet enzymes LipA and BioB, wherein sulfide from a second cluster is inserted into

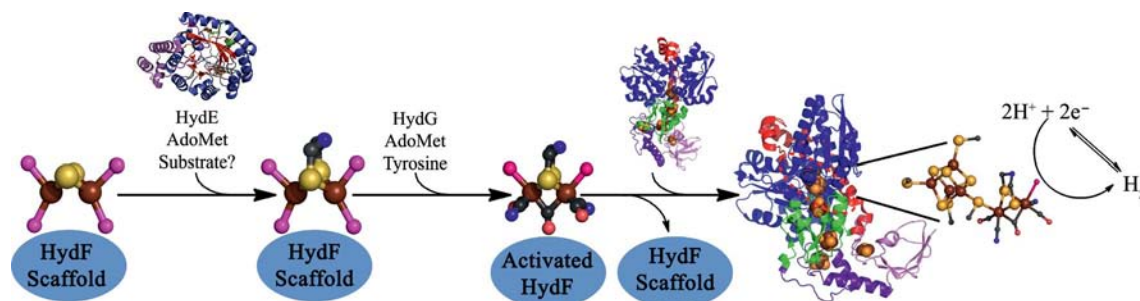


Figure 8. Maturation scheme for the [FeFe]-hydrogenase. Ball and stick models represent the intermediates in the biosynthesis of the H-cluster, with unknown ligands in magenta, carbon atoms in gray, oxygen in red, nitrogen in blue, sulfur in yellow, and iron in rust brown. The [2Fe-2S] precursor for the [2Fe] subcluster is thought to reside on HydF. The radical AdoMet enzyme HydE is thought to act upon the scaffold to synthesize the dithiolate bridge. HydG then uses tyrosine to produce the bound CO and CN-ligands of the subcluster. This primes the [2Fe] subcluster for transport and insertion into the HydA enzyme.

C–H bonds.<sup>[115–121]</sup> This second cluster could be the [2Fe-2S] cluster observed in the crystal structure of HydE from *Thermatoga maritima*;<sup>[97]</sup> alternatively, we have proposed that the cluster that serves as a sulfur source for the HydE-catalyzed reaction actually resides on HydF rather than on HydE (Figure 8).<sup>[89]</sup>

Our current working model for H-cluster assembly is thus that HydE acts on a [2Fe-2S] cluster bound to HydF, as well as an unknown organic substrate, to insert both bridging sulfides into C–H bonds of substrate to generate the bridging dithiolate ligand. This modified [2Fe] cluster on HydF is thus primed to accept the diatomic ligands synthesized by HydG to generate the [2Fe] H-cluster precursor containing the unique nonprotein ligands. Given the general biochemical toxicity of CO and CN<sup>−</sup>, it is likely that delivery of these diatomic ligands is tightly controlled, most likely by direct delivery in a HydG:HydF protein complex. Indeed, this model predicts the association of HydF with both HydE and HydG in a sequential manner, and biochemical evidence from our laboratories supports the hypothesis that these proteins interact.<sup>[87]</sup>

## 7.2 Prebiotic Implications of H-Cluster Biosynthesis

Recent work on radical-based CN<sup>−</sup> production during H-cluster biosynthesis has potentially far-reaching implications for prebiotic synthesis. A source of purines and nitrogenous compounds has been a stumbling point for many theories on the origin of life, however these become accessible in the presence of CN<sup>−</sup> due to the propensity for CN<sup>−</sup> polymerization.<sup>[122]</sup> Joyce et al. has suggested that purine nucleobases derived from HCN may have been the first nitrogenous information storage structures.<sup>[123]</sup> RNA itself could be abiotically produced if a source of stabilized ribose were available.<sup>[124,125]</sup> Yet, current theories suggest that the availability of HCN for polymerization could be limiting on pre-biotic earth. HCN does form readily, but generally it forms in settings not copacetic with other biologically relevant molecules.<sup>[126]</sup> Some non-radical-based theories for HCN synthesis on pre-biotic earth exist, however most do

not address issues of building sufficient concentrations of CN<sup>−</sup> for polymerization.<sup>[127]</sup> Recent work on H-cluster biosynthesis presented herein suggests radical-based creation of HCN could be a plausible mechanism for CN<sup>−</sup> creation on the hadean earth.<sup>[122]</sup> If these radical-based reactions occurred within the confines of a porous hydrothermal vent system, then it is conceivable that concentrations of HCN created would be high enough to polymerize many important pre-biotic molecules.<sup>[122]</sup>

## 8 Summary

Cyanide- and carbon monoxide-yielding reactions constitute a paradoxical story in biology; while both are potent metabolic poisons for primary biochemical processes, they also have a number of biologically relevant roles. In hydrogenase active sites they are essential building blocks in tailoring a catalytically active hydrogenase cofactor. The past ten years of research has been fruitful in identifying biogenic sources of cyanide and carbon monoxide in hydrogenase maturation of [NiFe]- and [FeFe]-hydrogenases. Identification of cyanide's differential metabolic origin in the [NiFe]- and [FeFe]-hydrogenase maturation processes (as defined by their substrates) is reflective of convergent methods for cyanide and carbon monoxide biosynthesis.

The recent research on the [FeFe]-hydrogenase maturase HydG has revealed a unique catalytic reaction in which CO and CN<sup>−</sup> are simultaneously synthesized. The HydG-catalyzed reaction represents a mode for synthesizing CO and CN<sup>−</sup>, and it is unlikely to be similar to the divergent [NiFe]- or [Fe]-hydrogenase maturation systems. While the exact mechanism has yet to be elucidated, our recent results demonstrate that radical chemistry is central to the synthesis of CN<sup>−</sup> and CO for the [FeFe]-hydrogenase active site.

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