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Journal

Dalton Transactions, 50(36)

ISSN

1477-9226

Authors

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Publication Date

2021-09-21

DOI

10.1039/d1dt02258j

Peer reviewed

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Author manuscript

Dalton Trans. Author manuscript; available in PMC 2022 September 21.

Published in final edited form as:

Dalton Trans. ; 50(36): 12386–12391. doi:10.1039/d1dt02258j.

Biosynthesis of the [FeFe] hydrogenase H-cluster *via* a synthetic [Fe(II)(CN)(CO)$_2$(cysteinate)]$^-$ complex

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Abstract

The H-cluster of [Fe–Fe] hydrogenase consists of a [4Fe]$_H$ subcluster linked by the sulfur of a cysteine residue to an organometallic [2Fe]$_H$ subcluster that utilizes terminal CO and CN ligands to each Fe along with a bridging CO and a bridging SCH_2NHCH_2S azadithiolate (adt) to catalyze proton reduction or hydrogen oxidation. Three Fe–S "maturase" proteins, HydE, HydF, and HydG, are responsible for the biosynthesis of the [2Fe]$_H$ subcluster and its incorporation into the hydrogenase enzyme to form this catalytically active H-cluster. We have proposed that HydG is a bifunctional enzyme that uses *S*-adenosylmethione (SAM) bound to a [4Fe–4S] cluster to lyse tyrosine *via* a transient 5′-deoxyadenosyl radical to produce CO and CN ligands to a unique cysteine-chelated Fe(II) that is linked to a second [4Fe–4S] cluster *via* the cysteine sulfur. In this "synthon model", after two cycles of tyrosine lysis, the product of HydG is completed: a [Fe(CN)(CO)$_2$(cysteinate)]$^-$ organometallic unit that is vectored directly into the synthesis of the [2Fe]$_H$ sub-cluster. However our HydG-centric synthon model is not universally accepted, so further validation is important. In this Frontiers article, we discuss recent results using a synthetic "Syn-B" complex that donates [Fe(CN)(CO)$_2$(cysteinate)]$^-$ units that match our proposed HydG product. Can Syn-B activate hydrogenase in the absence of HydG and its tyrosine substrate? If so, since Syn-B can be synthesized with specific magnetic nuclear isotopes and with chemical substitutions, its use could allow its enzymatic conversions on the route to the H-cluster to be monitored and modeled in fresh detail.

The [FeFe] hydrogenases contain an active site "H-cluster" that catalyzes the redox interconversion of protons and electrons with molecular hydrogen. These [Fe–Fe] hydrogenases and their [Ni–Fe] counterparts have generated much interest for renewable energy applications.[1–4] The H-cluster consists of a standard [4Fe–4S] cluster (denoted [4Fe]$_H$) linked to a unique binuclear [2Fe]$_H$ subcluster *via* a bridging cysteine (Fig. 1). The H^+ and H_2 substrates bind and react at the [2Fe]$_H$ unit,[5–8] which contains the organometallic elements of the H-cluster, with the two irons exhibiting CO and CN terminal ligands, and with two bridges linking these two irons in the form of a third CO along with a unique

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Conflicts of interest

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SCH_2NHCH_2S azadithiolate (adt) species (Fig. 1). Interesting questions in the biosynthesis of the H-cluster include the timing of the installation of CO and CN-ligands, assembly of the adt cofactor, especially since the free dithiol is unstable,[9] and the emergence of an initial Fe_2 pair.

It has long been known that the $[2Fe]_H$ subcluster is synthesized and linked to the $[4Fe]_H$ subcluster to form the active H-cluster by a set of three Fe–S proteins, HydE, HydF, and HydG (Fig. 1).[10–16] However two or even all three of these can be bypassed if a synthetic binuclear cluster precursor is introduced to the apo-hydrogenase lacking the H-cluster,[17,18] providing an interesting semi-synthetic route to hydrogenase activation.

Two of the three Fe–S maturases, HydE and HydG, belong to the radical SAM (*S*-adenosylmethionine) family of enzymes, which contain a site differentiated [4Fe–4S] cluster, where one iron has an open coordination position to which SAM binds. A 5′-deoxyadenosyl (5′dAdo$^\bullet$) radical is generated by 1-electron reduction of the [4Fe–4S] cluster which leads to a homolytic cleavage between the S-atom of methionine and the C5′ of adenosine. In the majority of rSAM enzymes, including HydG, this 5′dAdo$^\bullet$ abstracts a specific H-atom from the enzyme's substrate to generate a substrate-centered radical and dAdoH. In the case of HydG, the substrate is tyrosine, and this radical chemistry drives its fragmentation and leads in some fashion to the Fe-bound CN and CO ligands of the H-cluster, with different models asto how the latter chemistry occurs.

An early and oft-discussed model for the origin of the $[2Fe]_H$ subcluster proposes that it is built upon extant [2Fe–2S] cluster on HydF, (a GTPase), with the addition of "free" CN^- and CO provided by HydG and the adt bridge provided by the other radical SAM enzyme, HydE.[16,20–22] This model is obviously appealing since it provides a simple origin of the Fe_2S_2 core of $[2Fe]_H$, as such binuclear Fe–S clusters are produced by conventional Fe–S assembly machinery. As we discuss below, our experiments point to an alternative pathway, one that proceeds *via* $FeS(CO)_2CN$ modules.

A combination of cell-free synthesis, spectroscopic characterization of reaction intermediates, and isotopic labeling has ushered in a new model for the biosynthesis of the $[2Fe]_H$ subcluster. A key step in the development of this model, which we call the Complex B Pathway, came from recognition that HydG is bifunctional: two rather different processes occur within HydG. Its bifunctional nature is reflected by the presence of two Fe–S clusters, which are quite distant (24 Å).[23] One [4Fe–4S] cluster drives the rSAM reaction and catalyzes production of dehydroglycine (DHG) from tyrosine. The second cluster functions as a scaffold for the assembly of $[Fe(CN)(CO)_2(cysteinate)]^-$, called Complex B.[24,25]

The processes associated with the rSAM module of HydG have been well described,[26–28] but the processes at the second cluster merit discussion. The second cluster incorporates a [4Fe–4S] core with four thiolate ligands. Unlike, however, the canonical [4Fe–4S clusters], one of these thiolates is cysteine, not a cys residue. With a free amine and free carboxylate, this site provides an S,N,O facial chelating group that is well suited to bind a fifth "dangler" Fe as shown in Fig. 2.[23,25] This binding site is complemented by a conserved histidine residue. This Fe (S,N,O,his) center accumulates a CO ligand and a cyanide derived from

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the DHG to give Complex A.[24,25,29] In so doing, the dangler Fe changes from high- to low-spin, characteristic of ferrous carbonyl complexes.[30] A second equivalent of DHG converts Complex A to Complex B, a $(CO)_2$ complex which dissociates from the cubane, the broken Fe–S bond is replaced by the second cyanide, resulting in a $[Fe(CN)(CO)_2(cysteinate)]^-$. This "synthon" is low spin Fe(II) and hence $S = 0$ and is itself not observable by EPR, although its two CO modes and one CN mode are readily detected *via* FTIR as Complex B.

Aspects of this synthon model have come into question in publications favoring aspects of the original model for the synthesis of the H-cluster.[31,32] Here we examine the results on new experiments employing a fully synthetic analog of the proposed HydG $[Fe(CN)(CO)_2(cysteinate)]^-$ product. Can this synthetic synthon, termed "Syn-B", provide for [Fe–Fe] hydrogenase activation in the absence of HydG? If so, can more details of the H-cluster biosynthesis process be gleaned, for example with chemically modified Syn-B, or Syn-B generated with nuclear spin isotopes?

1 A synthetic $[Fe(CN)(CO)_2(cysteinate)]^-$ donor activates H-cluster synthesis in the absence of HydG

Our proposed $[Fe(CN)(CO)_2(cysteinate)]^-$ product of the dual enzymatic action of HydG provided an intriguing synthetic target for the Rauchfuss laboratory. They developed a synthetic carrier of this proposed synthon termed "Syn-B".[33] As noted, the use of synthetic precursors has been used successfully in the artificial maturation of apo-HydA with synthetic precursors to the $[2Fe]_H$ subcluster.[17,18] Generally speaking, synthetic precursors are applicable to reconstitution of enzymes when the module is lightly attached to the protein, *e.g.*, through a single donor-acceptor bond and hydrogen-bonding.

Our attempted synthesis of Complex B targeted the binding of cysteine to sources of "$Fe(CN)(CO)_x^+$". Since cationic iron cyanides are rare or unknown, we generated $[Fe(CN)I_2(CO)_3]^-$ by iodination of $[Fe(CN)(CO)_4]^-$. $[Fe(CN)I_2(CO)_3]^-$, which is labile, was treated with alkali metal salts of cysteinate in the hopes of obtaining Complex B. The ensuing chemistry is complicated but the end-result was still very positive. The dipotassium salt of cysteine, where the carboxylate and thiol are both deprotonated, reacts readily with $[Fe(CN)I_2(CO)_3]^-$ to produce compounds with IR signatures very close to that of Complex B. The product, which we call "Syn-B", is paramagnetic however. We propose that Syn-B consists of multiple copies of $[Fe(CN)I(CO)_2(cysH)]^-$ bound to a central high-spin ferrous Lewis acid *via* thiolate bridges. This proposal is consistent with the affinity of Complex-A for the high-spin Fe center of its [4Fe–4S] cluster. Terminal thiolate ligands, especially in anionic complexes, are "sticky". A number of lines of evidence are consistent with this description, including elemental analysis and ^{57}Fe Mössbauer studies (unpublished) on Syn-B that reveal that it contains a mixture of low and high spin Fe(II) centers.

In the maturation of the [Fe–Fe] hydrogenase H-cluster, HydG and tyrosine are absolutely required for hydrogenase activity.[34] However replacing HydG and tyrosine with Syn-B provides high activity, quite comparable to that of the conventional maturation using all three maturases, HydE, HydF, and HydG. As a control, attempted maturation with the cysteamine (cysamH = $H_2NCH_2CH_2SH$) analog of Syn-B provides no activity. In addition,

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the H-cluster EPR signals and ^{13}C CN ENDOR obtained *via* ^{13}CN-labeled Syn-B are identical to those of the conventionally maturated H-cluster. Thus we find that using a fully synthetic version of our proposed HydG synthon in HydG-less biosynthesis affords a catalytically active H-cluster with the appropriate electronic structure. In this important fashion Syn-B thus fullfills the same function as the well-characterized dinuclear iron cluster in H-cluster semisynthesis,[17,18] but now starting earlier at the mononuclear Fe level.

2 Syn-B incorporating magnetic isotopes or chemical substitutions provides details of the molecular sourcing of the H-cluster

Prior cell free synthesis using HydG and ^{13}C-tyrosine isotopologs has been used to ^{13}C-label and characterize the hyperfine couplings to the CN and CO ligands of the H-cluster,[36,37] and as noted above analogous studies with labeled Syn-B incorporated into HydG-less synthesis provides an H-cluster with identical electronic structure.[33] One limitation to our prior cell free synthesis was the inability to robustly isolate HydG lacking the exogenous cysteine bound at the auxiliary cluster and therefore the ability to replace this natural abundance cysteine with isotopically labeled cysteine, or alternatively, selenocysteine. Now, by synthesizing an analogue of Syn-B with selenocysteine in place of cysteine, we were able to test whether the bridging chalcogenide (S, Se) of the $[2Fe]_H$ subcluster are derived from the cysteine/selenocysteine of the synthon. Here Fe and Se edge EXAFS was used to confirm that sulfur/selenium of this cysteine/selenocysteine is vectored into the two adt sulfur/selenium bridges of the $[2Fe]_H$ subunit as incorporated into the fully active H-cluster. The rest of the atoms of the cysteine chelating the dangler Fe in HydG are eliminated, evidenced by the lack of ^{13}C and ^{15}N-pulse EPR signals when using $^{13}C_3{}^{15}N$-cysteine-Syn-B and more specifically by the concomitant appearance of $^{13}C_3$-pyruvate in parallel mass spectroscopy assays. This work provided another key line of evidence for the importance of HydG and its proposed synthon product: the Fe–S bond that originates in the cysteine-chelated dangler Fe(II) in HydG is retained throughout the full synthesis, including the intermediate Complex A of HydG,[29] the $Fe(II)(CO)_2(CN)$cysteine product of HydG, and into Fe_2S_2 core of the mature H-cluster. In further pulse EPR experiments, Rao *et al.*[29] demonstrated that the non-sulfur components of the adt bridge (CH_2NHCH_2) are derived from serine, specifically the amino group and the C3 methylene. Thus the molecular sourcing of each atom in the $[2Fe]_H$ subcluster is now determined, as shown in Fig. 3, with all but the CH_2NHCH_2 components of the bridge introduced *via* the HydG synthon.[19,35]

3 EPR spectroscopy reveals that Syn-B sourced $[Fe(CN)(CO)_2(cysteinate)]^-$ acts as the substrate of the second radical SAM enzyme, HydE

Early in the studies of the maturase enzymes, once it was established that the CO and CN ligands resulted from HydG,[20] it was suggested that the second radical SAM enzyme, HydE, is responsible for synthesis of the adt bridge.[16,22] A more complete characterization of the mechanism of HydE was delayed because both the substrate and the product of this maturase were unknown. Given the central role proposed for the HydG-derived synthon, as described above and summarized in Fig. 3, it seemed possible that this relatively complex $[Fe(CN)(CO)_2(cysteinate)]^-$ species could be the substrate for HydE. Tao *et al.*[38] tested

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this hypothesis with EPR spectroscopy. In samples frozen 10 s after reaction initiation one observes an EPR spectrum of an adenosylated Fe(I) intermediate (Fig. 4). By 10 min reaction time the cysteine C–S bond is cleaved exactly as observed in the full cell free synthesis, resulting in an altered Fe(I) EPR signal that we modeled as a $Fe(I)(CO)_2(CN)S$ core still coupled to the adenosyl moiety. This EPR signal loses intensity after 10 min, with one possibility that it forms an antiferromagnetically coupled $Fe(I)_2S_2(CO)_4(CN)_2$ dimer with an $S = 0$ ground state (dashed box). This is speculative, but suggests a possible HydE product, a proto-$[2Fe]_H$ dimer which is transferred to HydF for completion, with the adt bridge, sourced from serine, installed by enzymatic processes yet defined.

4 X-ray crystallography of HydE reveals the binding site of Syn-B sourced $[Fe(CN)(CO)_2(cysteinate)]^-$ and new clues to its activation

Following up on earlier X-ray structures showing that the 5′dAdo• generated by HydE forms S-adenosyl-cysteine adducts with thiazolidine compounds,[39] Nicolet *et al.*[40] have explored the crystallography of *Thermotoga maritima* HydE's interaction with Syn-B. The Syn-B-donated $[Fe(CN)(CO)_2(cysteinate)]^-$ is well resolved crystallographically and bound adjacent to a (*S*)-adenosyl-L-homocysteine (SAH) used as a nonreactive analog of SAM. Moreover, after crystallizing HydE following initiation of the reaction with SAM and Syn-B, X-ray structures reveal a new Fe centered species located at the other end of the internal HydE beta-barrel. This complex appears to be an 5-coordinate $Fe(CO)_2(CN)Cl$ species interacting with a conserved methionine(244). It is tempting to speculate that the related methionine-stabilized $Fe(CO)_2(CN)SH$ entity would be a biosynthetic intermediate. Thus this new X-ray structural work both reinforces the conclusions of the prior HydE-Syn-B EPR study and provides intriguing new structural details.

5. Conclusion

HydG-less maturation of the active H-cluster using the $[Fe(CN)(CO)_2(cysteinate)]^-$ donor Syn-B strongly supports the synthon model in which HydG injects such a mononuclear organometallic species into the H-cluster biosynthesis. In this synthon model, both radical SAM enzymes HydG and HydE play bifunctional roles in the synthesis of the H-cluster. Interestingly, the various Fe–S clusters involved in the biosynthesis are generally site-differentiated. All of the ligands of the $[2Fe]_H$ cluster are derived from amino acids. The HydE radical SAM mechanism for degrading cysteine to form the proposed $Fe(I)_2S_2(CO)_4(CN)_2$ dimer intermediate is novel.

Remaining major questions include: Is the dimer core of the $[2Fe]_H$ subcluster initially formed on HydE, as suggested? And how are two serines enzymatically processed to add the CH_2NHCH_2 component to the cysteine derived sulfurs that anchor the adt bridge? Does this reaction directly involve HydF, the only Fe–S maturase that is not a radical SAM enzyme?

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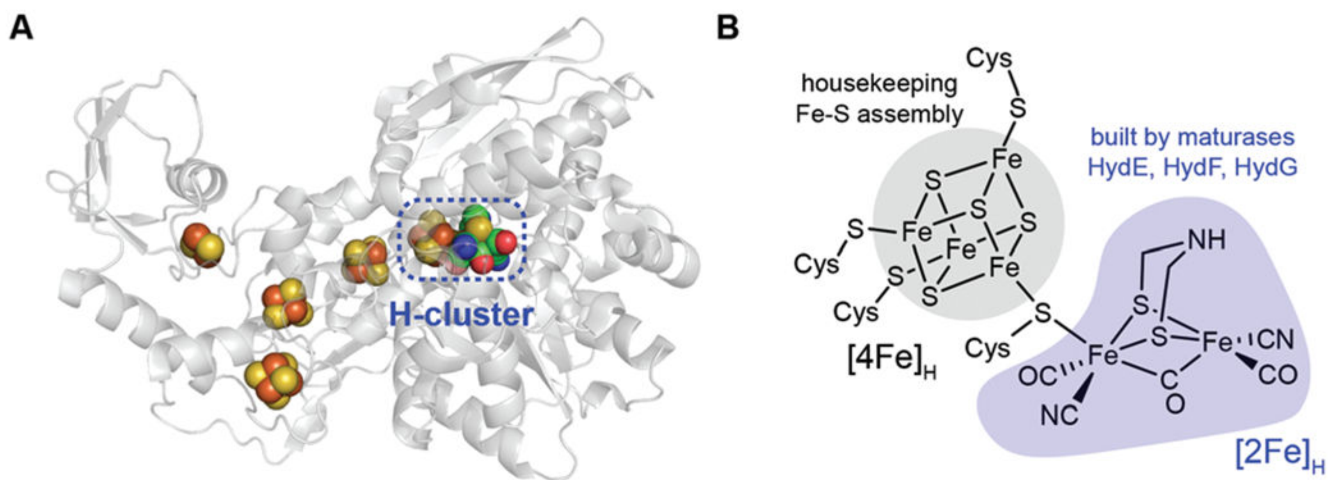

Fig. 1.

The [FeFe] hydrogenase and its active site. (A) X-ray structure of *Clostridium pasteurianum* CpI (PDB ID: 4XDC) highlighting the H-cluster and accessory Fe–S clusters serving as electron transfer wires. (B) Structure of the catalytic H-cluster, with the subclusters $[4Fe]_H$ and $[2Fe]_H$ that are assembled by different pathways in cells. Figure from ref. 19.

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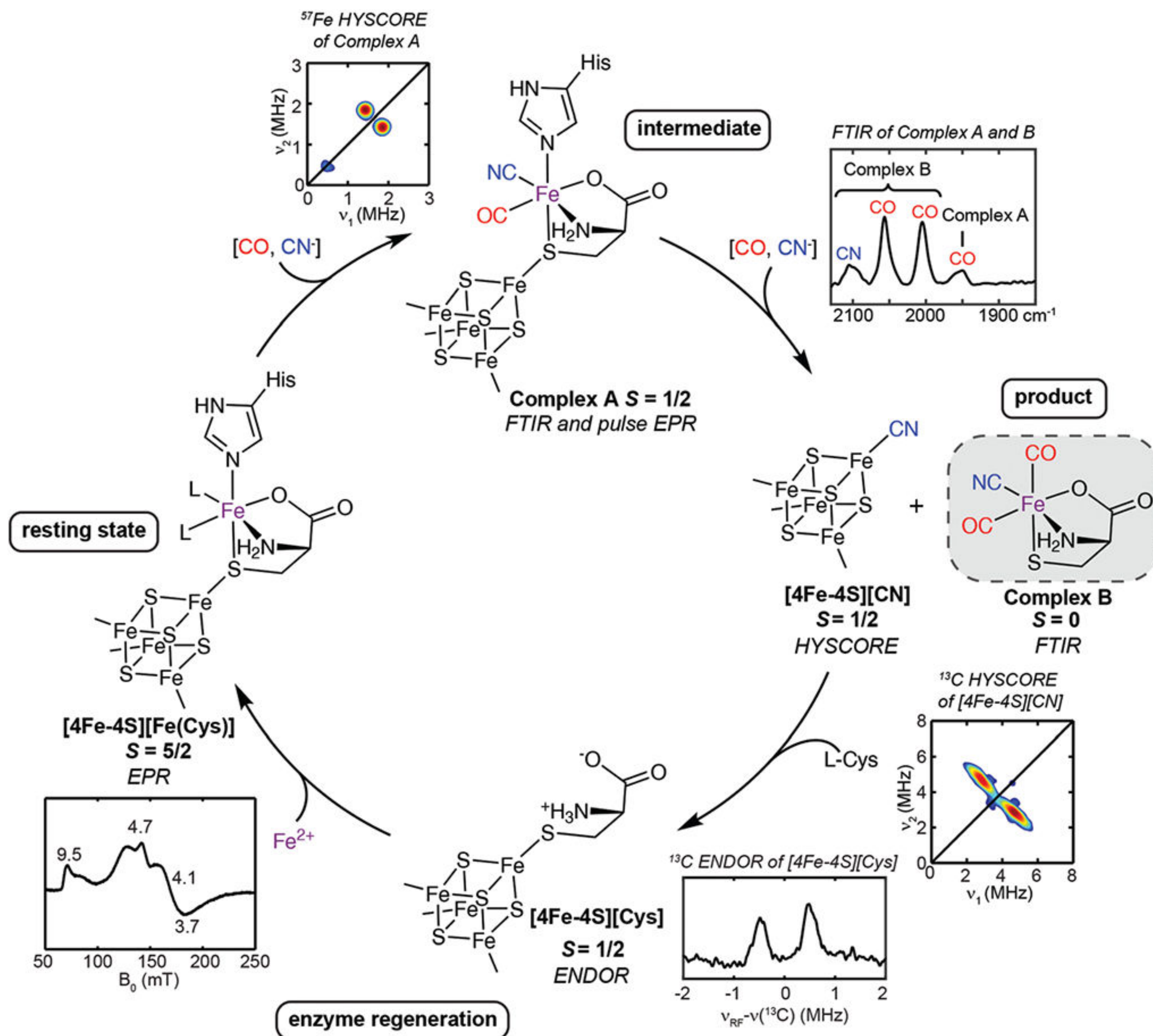

Fig. 2.

The catalytic cycle of HydG to generate the [Fe(CO)$_2$(CN)(cysteinate)] synthon, along with the reaction intermediates and their corresponding spectroscopic features. Figure from ref. 19.

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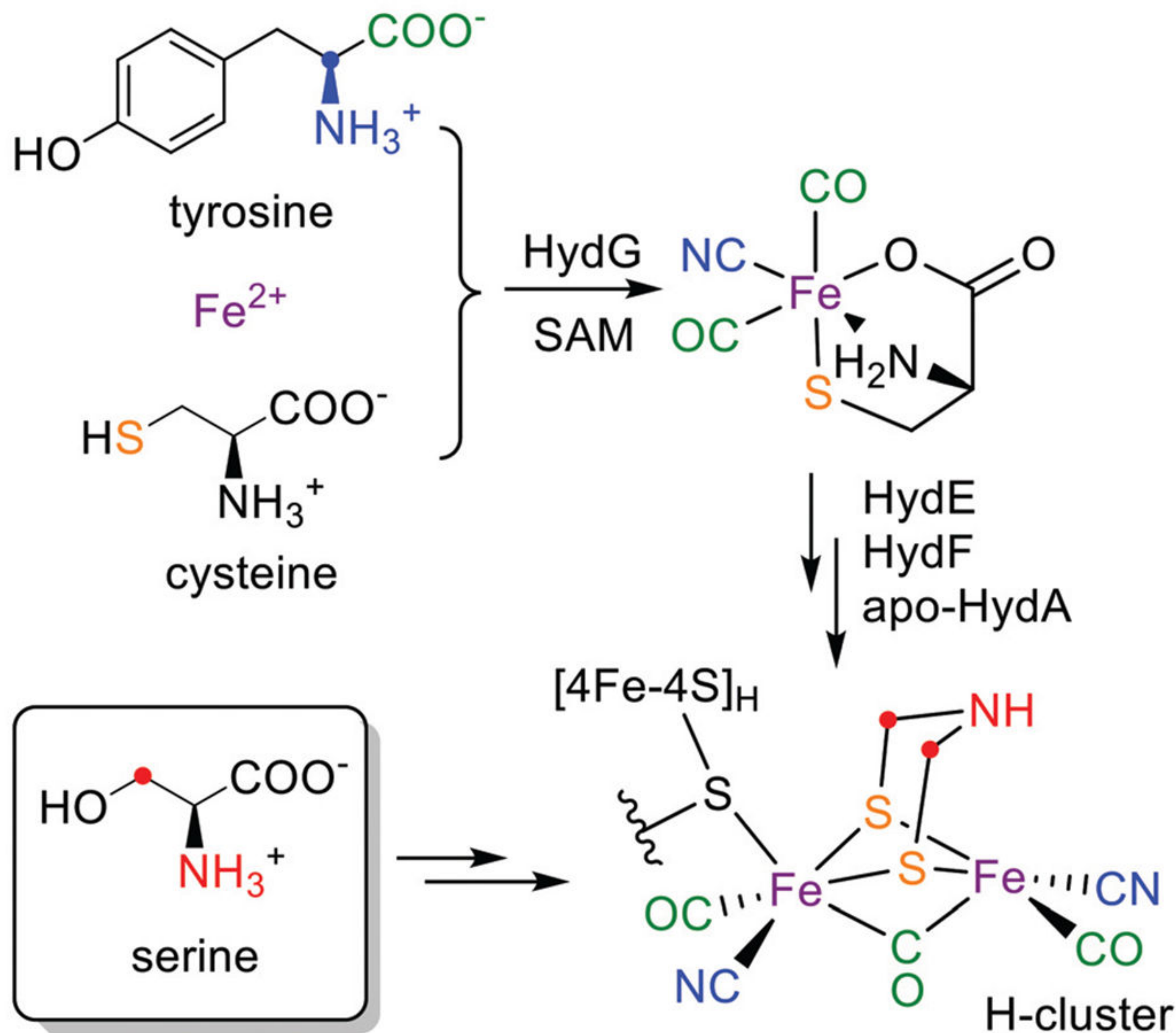

Fig. 3.

The HydG synthon provides the molecular origins of all atoms in the $[2Fe]_H$ subcluster other then the C and N atoms of the adt bridge which are derived from serine. Figure adapted from ref. 35.

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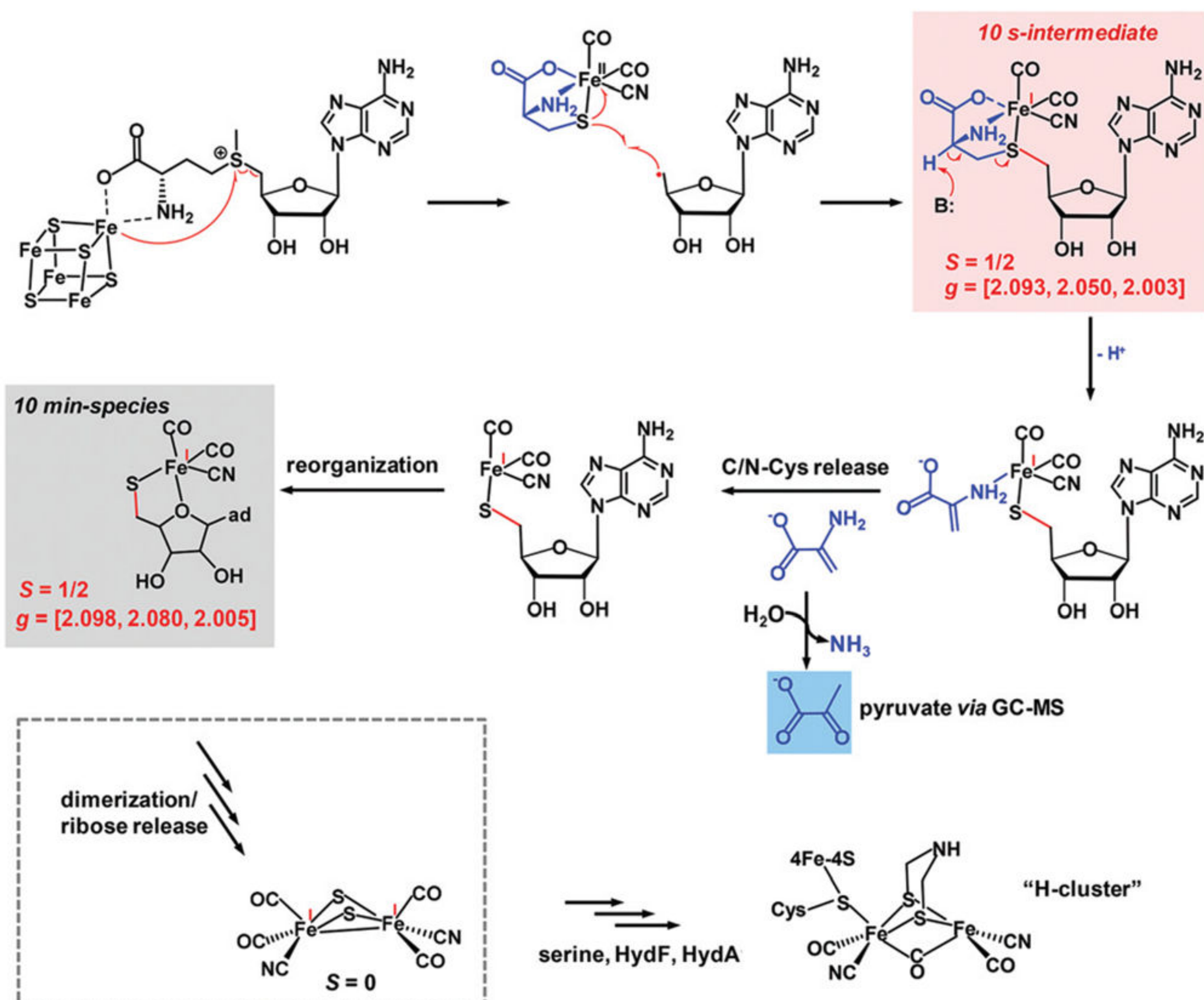

Fig. 4.

Overview of our proposed reaction pathway of HydE reacting with the HydG product $[Fe(CN)(CO)_2(cysteinate)]^-$ synthon. From: ref. 38.