Elsevier Editorial System(tm) for Current Opinion in Structural Biology

Manuscript Draft

Manuscript Number: COSTBI-D-16-00060R1

Title: Metallocofactor Assembly for [FeFe]-Hydrogenases.

Article Type: 41 Catalysis and Regulation 2016

Corresponding Author: Dr. Peter Roach,

Corresponding Author's Institution:

First Author: Pedro Dinis

Order of Authors: Pedro Dinis; Beata M Wieckowski; Peter Roach

Abstract: Hydrogenases are a potential source of environmentally benign bioenergy, using complex cofactors to catalyze the reversible reduction of protons to form hydrogen. The most active subclass, the [FeFe]-hydrogenases, is dependent on a metallocofactor, the H cluster, that consists of a two iron subcluster ([2Fe]H) bridging to a classical cubane cluster ([4Fe-4S]H). The ligands coordinating to the diiron subcluster include an azadithiolate, three carbon monoxides, and two cyanides. To assemble this complex cofactor, three maturase enzymes, HydG, HydE and HydF are required. The biosynthesis of the diatomic ligands proceeds by an unusual fragmentation mechanism, and structural studies in combination with spectroscopic analysis have started to provide insights into the HydG mediated assembly of a [2Fe]H subcluster precursor.

Conflict of interest statement

Nothing declared.

***Highlights**

- Crystallography identifies a [5Fe-5S] auxiliary cluster in HydG including a labile iron site.
- The HydG labile iron is proposed as the site of $Fe(CO)_2CN$ synthon biosynthesis
- Spectroscopy demonstrates the cysteine dependent formation of a $Fe(CO)_2CN$ synthon.
- Synthetic [2Fe] mimics provide a concise route to fully active [FeFe]-hydrogenases

Metallocofactor Assembly for [FeFe]-Hydrogenases.

Pedro Dinis, Beata M. Wieckowski and Peter L. Roach.

Addresses

Chemistry and the Institute for Life Sciences, University of Southampton, Highfield Campus, Southampton SO17 1BJ, UK.

Corresponding author: Peter L. Roach (plr2@soton.ac.uk).

Abstract

Hydrogenases are a potential source of environmentally benign bioenergy, using complex cofactors to catalyze the reversible reduction of protons to form hydrogen. The most active subclass, the [FeFe]-hydrogenases, is dependent on a metallocofactor, the H cluster, that consists of a two iron subcluster ($[2Fe]_H$) bridging to a classical cubane cluster ($[4Fe\text{-}4S]_H$). The ligands coordinating to the diiron subcluster include an azadithiolate, three carbon monoxides, and two cyanides. To assemble this complex cofactor, three maturase enzymes, HydG, HydE and HydF are required. The biosynthesis of the diatomic ligands proceeds by an unusual fragmentation mechanism, and structural studies in combination with spectroscopic analysis have started to provide insights into the HydG mediated assembly of a $[2Fe]_H$ subcluster precursor.

Introduction

The reversible reduction of protons to yield hydrogen is efficiently catalyzed by [FeFe]-hydrogenases [1]. At the core of this catalytic activity is a cofactor, the H-cluster, an organometallic center made up of two subclusters. The $[4Fe\text{-}4S]_H$ subcluster is a rather conventional cubane, held in place by four cysteine residues [2-4]. (Herein we will identify clusters with subscripts, for example $[2Fe]_H$, $[4Fe\text{-}4S]_{RS}$ or $[4Fe\text{-}4S]_{Aux}$ respectively to indicate subclusters of the H cluster, clusters involved in radical S-adenosylmethionine (SAM) (RS) chemistry or with auxiliary functions respectively). In contrast, the $[2Fe]_H$ subcluster is held by a single protein ligand, a cysteine residue, but contains three carbon monoxides, two cyanides and an azadithiolate ligand [5]. The contrast between the sub-clusters extends to their assembly (Figure 1a), as the $[4Fe\text{-}4S]_H$ subcluster is synthesized and inserted by general Fe-S cluster biosynthetic machinery, however biosynthesis and insertion of the $[2Fe]_H$ subcluster requires the activity of highly specific maturase enzymes, HydE, HydG and HydF [6].

Sequence analysis of HydG suggested a close relationship to a radical SAM enzyme, ThiH, which cleaves tyrosine to yield *p*-cresol and dehydroglycine [7,8]. Enzymes of the radical SAM superfamily share a common mechanism of radical reaction initiation, using a $[4Fe\text{-}4S]^+$ cluster to reductively cleave SAM, which yields methionine and the 5′-deoxyadenosyl radical, a highly reactive primary radical [9] (Figure 1b). Usually radical SAM enzymes use this intermediate to initiate radical rearrangements by abstraction of hydrogen from a second substrate; in the case of HydG, abstraction from tyrosine [10]. Structural studies with a related radical SAM enzyme [11] suggest that in HydG the hydrogen atom is abstracted from the α-nitrogen of tyrosine, followed by β-scission

of the tyrosine Cα-Cβ bond (Figure 1c). The resultant hydroxybenzyl radical has been characterized in detail using tyrosine isotopologues and freeze quench EPR spectroscopy [12]. Biochemical experiments with HydG identified three tyrosine derived products: the byproduct *p*-cresol [7], cyanide [13] and carbon monoxide [14]. The last two are incorporated into the $[2Fe]_H$ subcluster [15] together with HydG-derived iron atoms [16]

The intermediate dehydroglycine is converted to CO and cyanide, a reaction that likely involves a second FeS cluster present in HydG, termed the auxiliary cluster [17]. The binding of these ligands to the auxiliary cluster was first reported using time resolved FTIR spectroscopy, which identified two further intermediates (Figure 1 c), complex A, formed after ~30 seconds, which is subsequently converted to complex B [12]. The presence of a second CO ligand in complex B implies a likely requirement for the fragmentation of two dehydroglycine equivalents to form this intermediate.

The two FeS clusters present in HydG have been subjected to detailed spectroscopic analysis. The N-terminal cluster consistently showed EPR properties typical of the cluster used for radical SAM chemistry, $[4Fe\text{-}4S]_{RS}$ [12,17,18], however more variation in the spectroscopic properties for the C-terminal auxiliary cluster have been reported. It seems likely that differences in the enzyme source, purification method and whether the clusters were subject to chemical reconstitution (addition of exogenous Fe(II) and sulfide after purification) contributed to these spectroscopic discrepancies. For example, the auxiliary clusters of HydGs from *Clostridium acetobutylicum* (*Ca*) [17] and *Thermotoga maritima* (*Tm*) [18] have spectroscopic properties of a $[4Fe\text{-}4S]^+$ cluster, whereas EPR analysis of the enzyme from *Shewanella oneidensis* (*So*) upon isolation contained predominantly a high spin FeS auxiliary cluster that upon turnover in the presence of tyrosine, SAM and dithionite converted to a $[4Fe\text{-}4S]^+$ form [12].

Structure of HydG.

Two HydG crystal structures have been described [19,20] but the structure of the enzyme from *Thermoanaerobacter italicus* (*Ti*HydG) is the first reported with an intact auxiliary structure (Figure 2). The asymmetric unit contains two HydG monomers and the core structure of HydG is a complete triose phosphate isomerase (TIM) barrel, typical of RS enzymes the bind a small second substrate (such as PylB [21], BioB [22] and NosL [11]) (Figure 2a). The RS and auxiliary clusters are bound at either end of the TIM barrel, separated by more than 23 Å (Figure 2b) and, unusually for RS enzymes, HydG includes a C-terminal helical domain (80 amino acid residues) that encloses the auxiliary cluster (Figure 2a).

The Fe-S clusters and ligands in the two monomers differ significantly, but an anomalous difference Fourier map calculated from data collected at the iron K edge provided a reliable method for locating the position of the iron atoms. The $[4Fe\text{-}4S]_{RS}$ clusters are embedded within canonical sequence motifs shared by members of the radical SAM family [23]. The $[4Fe\text{-}4S]_{RS}$ clusters of monomers A and B are coordinated by the α-amino and α-carboxy groups of methionine and SAM respectively. The SAM sulfonium sulfur atom is poised 3.4 Å from the proximal iron, an arrangement that closely resembles SAM binding in other radical SAM enzymes [23]. The structure of co-crystals of HydG with tyrosine have not yet been reported, but this complex has been modelled [20] with the tyrosine binding pocket adjacent to the 5'-C of SAM (Figure 2b).

Two discrete states of the auxiliary cluster are observed in the two HydG monomers. Monomer A contains a [5Fe-5S] cluster (Figure 2c) that can be considered a conventional [4Fe-4S] cubane linked via a bridging sulfide to the fifth iron atom. This fifth iron has an occupancy of 0.73 and

approximately octahedral geometry. It is held in place by a single bonding interaction to the protein, coordination from imidazole of His265, which is highly conserved in HydG sequences [20]. The four remaining ligands to the fifth iron include two water molecules and a nonproteinacous amino acid whose side chain was not sufficiently well ordered to permit unequivocal identification (although it is suspected to be methionine arising from SAM degradation during crystallization). An interesting contrast is provided by the auxiliary cluster in monomer B, which consists of the [4Fe-4S] cubane with density corresponding to a (hydro)sulfide, but the fifth iron is absent. The partial occupancy of the fifth iron in monomer A and complete absence in monomer B suggested it might be loosely bound. To capture this concept, we termed it the 'labile iron' and suggested it as a potential site for the assembly of the $Fe(CO)_2CN$ synthon which might be readily released to a downstream acceptor such as HydF for further elaboration into the $[2Fe]_H$ subcluster [20].

This mechanism requires the transfer of the dehydroglycine intermediate from the proximity of the $[4Fe\text{-}4S]_{RS}$ cluster down a channel that traverses the TIM barrel lengthwise (pale blue cavity, Figure 2b), narrowing to a pinch point formed by the auxiliary cluster. Several mechanisms can be envisaged for the fragmentation of dehydroglycine and one of the simpler possibilities (Figure 3a) can be accelerated by an active site base (B in Figure 3a) which may be provided by the sidechains of conserved Arg129 or Arg165 (*Ti*HydG numbering), and/or an electrophile (Figure 3a, E^+) which might be a proton from an acidic residue or a Lewis acid such as the labile iron. Using a *Ca*HydG mutant that has two cysteine residues that normally bind the auxiliary cluster mutated to serine, and therefore does not bind an intact auxiliary cluster, the formation of cyanide without CO formation has been measured [24] and this mutant produces formate in place of CO [25]. This implies that under these conditions, cleavage of dehydroglycine to form cyanide occurs prior to the conversion of the formyl moiety into carbon monoxide [25].

Further insight into the *in vivo* function of the auxiliary cluster has come from studies on the role of L-cysteine in HydG catalysis [26-28]. A combination of EPR and ENDOR spectroscopy demonstrated that L-Cys (but not D-Cys, L-homocysteine, alanine plus sulfide or L-serine) could bind to the auxiliary cluster, replacing the bridging sulfide ion and amino acid ligand observed in the *Ti*HydG structure (Figure 3b)[26]. Cysteine is likely incorporated during *So*HydG expression and remains intact during a mild affinity purification, or can be introduced by the addition of exogenous cysteine. This is proposed to yield a $[4Fe\text{–}4S]_{Aux}[(\kappa^3\text{-Cys})Fe]$ auxiliary cluster (Figure 3b, **1**). Moreover, upon turnover (addition of ^{13}C-Tyr, SAM and dithionite), the auxiliary cluster is converted via complex A and complex B to a cyanide derivative (Figure 3b, **4**), presumably with concomitant extrusion of the $[Fe(CO)_2CNCys]^-$ synthon (Figure 3b, **5**). In an effort to analyze the functional importance of the auxiliary cluster thiol ligand, HydG samples with different auxiliary cluster states have been prepared, with no labile iron, or including a bridging sulfide or cysteine or homocysteine as potential labile iron ligands. For these samples, the formation of HydG-bound CO and cyanide ligands has been measured by stopped flow IR and only the sample containing a labile iron bound to L-cysteine (Figure 3b, **1**) showed strong activity for turnover to form HydG-bound diatomic ligands [28]. Figure 3b incorporates these results into a mechanistic proposal for HydG.

HydE: 'a riddle, wrapped in a mystery, inside an enigma'.

HydE is a member of the RS family and is essential for [FeFe]-hydrogenase maturation [6,29]. Several years ago, structures of *Tm*HydE were reported from a crystal form that diffracted to high resolution (1.25 Å)[30]. These crystals have been used in a crystallographic study of the reductive cleavage of SAM [31]. Some HydE sequences contain an auxiliary cluster binding site, but it is of variable

constitution and can be removed by mutation without inactivating HydE, suggesting it is functionally inessential [30,32]. The structure of HydE (Figure 4a) includes a large cavity (991 $Å^3$) within the TIM barrel that is well suited to bind a second substrate (in addition to SAM). Efforts to identify this substrate include *in silico* [24] and *in vitro* screening experiments [32,33]. The rate of SAM turnover to form 5'-deoxyadenosine was recently used to screen a range of putative HydE second substrates, leading to the conclusion that the HydE substrate likely contains a thiol [33]. Thiocyanate was identified as a fragment with significant affinity for HydE [30] and more recent studies with HydE crystals demonstrated that a small molecule, (2*R,*4*R*)-2-methyl-1,3-thiazolidine-2,4-dicarboxylic acid, could intercept the 5'-deoxyadenosyl radical intermediate, forming a new 5'-C to sulfur bond in the product [32]. However, despite all of this hard won understanding, the natural substrate and products for HydE remain elusive. One putative role for HydE is catalyzing the biosynthesis of the azadithiolate ligand [34,35], but measuring the formation of this ligand is experimentally challenging, as in free aqueous solution such geminal hemithioaminal groups are inherently unstable and undergo hydrolysis (to formaldehyde, sulfide and ammonia in this case). Following the metabolic fate of ^{15}N labelled precursors through in vitro maturation may circumvent this problem [36].

HydF as a Scaffold for HydA Maturation.

HydF has been proposed to function as a template or scaffold for the assembly of the [2Fe] subcluster, which is then transferred as a unit to HydA [1, 2]. The only crystal structure of HydF (Figure 4b) was obtained under aerobic conditions and therefore lacks Fe-S clusters. However, each monomer of the *Thermotoga neapolitana* (*Tn*) HydF dimer consists of three domains, for GTP binding (I), dimerization (II) and cluster binding (III) which includes potential Fe-S cluster binding residues (3 Cys and 2 His, Figure 4b). Given the important role for HydF in the complex process of $[2Fe]_H$ subcluster assembly, it is perhaps unsurprising that the reported nature of the cluster(s) bound to HydF vary depending on the protein source, chemical reconstitution, heterologous or homologous expression, and co-expression with or without the other maturases (termed $HydF^{EG}$ and $HydF^{\Delta EG}$ respectively). There is good agreement that HydF binds a [4Fe-4S] cluster, but variability in the observed state of the second Fe-S cluster: for example, reconstituted *Tm*$HydF^{\Delta EG}$ and *Tn*$HydF^{\Delta EG}$ likely bear no second cluster [37,38], EPR spectra of unreconstituted *Ca*$HydF^{\Delta EG}$ shows a signal resembling a [2Fe-2S] cluster [39,40] and IR spectroscopy suggests multiple diatomic ligands bound to *Ca*$HydF^{EG}$ [40,41]. Models for cluster assembly by HydF agree that the [4Fe-4S] is assembled by housekeeping iron sulfur cluster biosynthetic enzymes. Two alternatives have been considered [34] for assembly of the [2Fe] subcluster: (i) housekeeping enzymes also insert a [2Fe-2S] cluster which is modified by ligands introduced by transfer from HydG/HydE or (ii) the [2Fe] subcluster is derived from two equivalents of the $Fe(CO)_2CN$ synthon produced by HydG. Although many unresolved questions remain, using a biosynthetic cocktail (including maturases, substrates and cofactors) to achieve efficient transfer of labelled ^{57}Fe from HydG to the $[2Fe]_H$ subcluster of HydA [16] supports model (ii). Direct interactions between the maturase proteins HydE and HydG with HydF have been demonstrated [29,39] and GTP has been shown to accelerate the dissociation of HydE:HydF and HydG:HydF complexes [40], although the significance of these interactions and the GTPase activity awaits more detailed functional analysis.

The optimal complete maturase cocktail is complex (including HydA, the maturases HydE, F and G, GTP, cysteine, PLP and an aliquot of *E. coli* cell lysate) [15], but a concise alternative approach to HydA maturation was provided by a semi-synthetic system, using biologically derived proteins and synthetic [2Fe] mimics (Figure 4c, **7**) [42]. Incubation of the synthetic [2Fe] mimic with *Tm*HydF purified in a form bearing only a [4Fe-4S] cluster resulted in incorporation of the mimic to yield a

[4Fe-4S][2Fe]HydF complex (Figure 4c, **8**) with spectroscopic properties similar to HydF isolated from a host expressing the full maturase machinery [41]. Detailed spectroscopic analysis confirmed the orientation of the bridging cyanide ligand. This HydF complex could be used to activate one of the hydrogenases of *Chlamydomonas reinhardtii, apo*HydA1, by transfer of the [2Fe] subcluster from HydF to the unoccupied subsite on *apo*HydA1 (Figure 4c, 9). This system was subsequently further simplified by the activation of the hydrogenase directly by the [2Fe] mimic without a requirement to include HydF [43]. Using a range of synthetic [2Fe] analogues demonstrated that only the natural azadithiolate ligand (Figure 4c, **7b**) yielded a vigorously active hydrogenase and suggested this semisynthetic route could be used to explore the activities of a wide range of [FeFe]-hydrogenases in combination with a range of $[2Fe]_H$ mimics.

Conclusions

For the initial steps of H-cluster biosynthesis, structural and spectroscopic analysis of HydG has provided important experimental support for a pathway to an organometallic $Fe(CO)_2CN$ synthon from which a $[2Fe]_H$ subcluster precursor may be biosynthesized. At the other end of the biosynthetic pathway, a synthetic [2Fe] precursor mimic can be loaded into HydF and subsequently transferred into HydA to yield the active *holo*-HydA. However, there are still many unresolved questions. How does HydG catalyze the fragmentation of dehydroglycine? Can further structural analysis illuminate the mode of cysteine binding to the labile iron, or the precise arrangement of diatomic ligands bound to the labile iron? What is the fate of the proposed $[(Cys)Fe(CO)_2(CN)]^-$ biosynthetic intermediate? Does this unstable intermediate require a direct protein-to-protein transfer step? How is the role of HydE tied into the formation of the azadithiolate bridge? What are the azadithiolate precursors? Experimental tests will ultimately provide answers for these and other questions, doubtless with a fair measure of interesting results and surprises along the way.

Acknowledgements

We thank the staff at the Diamond Light Source Synchrotron for assistance and access to Beamline I03 under Proposal 8889. Our research was supported by the University of Southampton, the Engineering and Physical Sciences Research Council (EPSRC) and the Biotechnology and Biological Sciences Research Council. P.D. holds an EPSRC Doctoral Prize grant.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

** of outstanding interest

References

1. Lubitz W, Ogata H, Rudiger O, Reijerse E: **Hydrogenases**. *Chem Rev* 2014, **114**:4081-4148.
2. Mulder DW, Boyd ES, Sarma R, Lange RK, Endrizzi JA, Broderick JB, Peters JW: **Stepwise [FeFe]-hydrogenase H-cluster assembly revealed in the structure of HydA(DeltaEFG)**. *Nature* 2010, **465**:248-251.
3. Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC: **X-ray crystal structure of the Fe-only hydrogenase (CpI) from Clostridium pasteurianum to 1.8 angstrom resolution.** *Science* 1998, **282**:1853-1858.

4. Nicolet Y, Piras C, Legrand P, Hatchikian CE, Fontecilla-Camps JC: **Desulfovibrio desulfuricans iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center.** *Structure/Folding and Design* 1999, **7**:13-23.
5. Nicolet Y, Lemon BJ, Fontecilla-Camps JC, Peters JW: **A novel FeS cluster in Fe-only hydrogenases**. *Trends Biochem Sci* 2000, **25**:138-143.
6. Posewitz MC, King PW, Smolinski SL, Zhang L, Seibert M, Ghirardi ML: **Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase**. *J Biol Chem* 2004, **279**:25711-25720.
7. Pilet E, Nicolet Y, Mathevon C, Douki T, Fontecilla-Camps JC, Fontecave M: **The role of the maturase HydG in [FeFe]-hydrogenase active site synthesis and assembly**. *FEBS Lett* 2009, **583**:506-511.
8. Kriek M, Martins F, Challand MR, Croft A, Roach PL: **Thiamine biosynthesis in Escherichia coli: identification of the intermediate and by-product derived from tyrosine**. *Angew Chem Int Ed Engl* 2007, **46**:9223-9226.
9. Hiscox MJ, Driesener RC, Roach PL: **Enzyme catalyzed formation of radicals from S-adenosylmethionine and inhibition of enzyme activity by the cleavage products**. *Biochim Biophys Acta* 2012, **1824**:1165-1177.
10. Duffus BR, Ghose S, Peters JW, Broderick JB: **Reversible H atom abstraction catalyzed by the radical S-adenosylmethionine enzyme HydG**. *J Am Chem Soc* 2014, **136**:13086-13089.
11. Nicolet Y, Zeppieri L, Amara P, Fontecilla-Camps JC: **Crystal Structure of Tryptophan Lyase (NosL): Evidence for Radical Formation at the Amino Group of Tryptophan**. *Angew Chem Int Ed Engl* 2014, **53**:11840-11844.
12. Kuchenreuther JM, Myers WK, Stich TA, George SJ, Nejatyjahromy Y, Swartz JR, Britt RD: **A radical intermediate in tyrosine scission to the CO and CN- ligands of FeFe hydrogenase.** *Science* 2013, **342**:472-475.
13. Driesener RC, Challand MR, McGlynn SE, Shepard EM, Boyd ES, Broderick JB, Peters JW, Roach PL: **[FeFe]-hydrogenase cyanide ligands derived from S-adenosylmethionine-dependent cleavage of tyrosine**. *Angew Chem Int Ed Engl* 2010, **49**:1687-1690.
14. Shepard EM, Duffus BR, George SJ, McGlynn SE, Challand MR, Swanson KD, Roach PL, Cramer SP, Peters JW, Broderick JB: **[FeFe]-hydrogenase maturation: HydG-catalyzed synthesis of carbon monoxide**. *J Am Chem Soc* 2010, **132**:9247-9249.
15. Kuchenreuther JM, George SJ, Grady-Smith CS, Cramer SP, Swartz JR: **Cell-free H-cluster Synthesis and [FeFe] Hydrogenase Activation: All Five CO and CN− Ligands Derive from Tyrosine**. *PLoS ONE* 2011, **6**:e20346.
16. Kuchenreuther JM, Myers WK, Suess DLM, Stich TA, Pelmenschikov V, Shiigi S, Cramer SP, Swartz JR, Britt RD, George SJ: **The HydG Enzyme Generates an Fe (CO) 2 (CN) Synthon in Assembly of the FeFe Hydrogenase H-Cluster**. *Science* 2014, **343**:424-427.

** This paper provided the first characterisation of a HydG-bound $Fe(CO)_2CN$ synthon using stopped-flow IR. A second incisive experiment identified HydG as the source of Fe for the $[2Fe]_H$ subcluster by using ENDOR spectroscopy to track a ^{57}Fe label from HydG into the mature H-cluster of *holo*-HydA

17. Driesener RC, Duffus BR, Shepard EM, Bruzas IR, Duschene KS, Coleman NJR, Marrison APG, Salvadori E, Kay CWM, Peters JW, et al.: **Biochemical and Kinetic Characterization of Radical S-Adenosyl-L-methionine Enzyme HydG**. *Biochemistry* 2013, **52**:8696-8707.
18. Rubach JK, Brazzolotto X, Gaillard J, Fontecave M: **Biochemical characterization of the HydE and HydG iron-only hydrogenase maturation enzymes from Thermatoga maritima**. *FEBS Lett* 2005, **579**:5055-5060.
19. Nicolet Y, Pagnier A, Zeppieri L, Martin L, Amara P, Fontecilla-Camps JC: **Crystal structure of HydG from Carboxydothermus hydrogenoformans: a trifunctional [FeFe]-hydrogenase maturase**. *Chembiochem* 2015, **16**:397-402.

20. Dinis P, Suess DL, Fox SJ, Harmer JE, Driesener RC, De La Paz L, Swartz JR, Essex JW, Britt RD, Roach PL: **X-ray crystallographic and EPR spectroscopic analysis of HydG, a maturase in [FeFe]-hydrogenase H-cluster assembly**. *Proc Natl Acad Sci U S A* 2015, **112**:1362-1367.

** This study correlated the crystallographic and EPR spectroscopic analysis of HydG, identifying the and characterising the labile Fe binding site. This was key to the development of a mechanistic proposal in which the labile iron is the likely site for $Fe(CO)_2CN$ synthon biosynthesis.

21. Quitterer F, List A, Eisenreich W, Bacher A, Groll M: **Crystal structure of methylornithine synthase (PylB): insights into the pyrrolysine biosynthesis**. *Angew Chem Int Ed Engl* 2012, **51**:1339-1342.
22. Berkovitch F, Nicolet Y, Wan JT, Jarrett JT, Drennan CL: **Crystal structure of biotin synthase, an S-adenosylmethionine-dependent radical enzyme**. *Science* 2004, **303**:76-79.
23. Vey JL, Drennan CL: **Structural insights into radical generation by the radical SAM superfamily**. *Chem Rev* 2011, **111**:2487-2506.
24. Nicolet Y, Martin L, Tron C, Fontecilla-Camps JC: **A glycyl free radical as the precursor in the synthesis of carbon monoxide and cyanide by the [FeFe]-hydrogenase maturase HydG**. *FEBS Lett* 2010, **584**:4197-4202.
25. Pagnier A, Martin L, Zeppieri L, Nicolet Y, Fontecilla-Camps JC: **CO and CN- syntheses by [FeFe]-hydrogenase maturase HydG are catalytically differentiated events**. *Proc Natl Acad Sci U S A* 2016, **113**:104-109.
26. Suess DL, Burstel I, De La Paz L, Kuchenreuther JM, Pham CC, Cramer SP, Swartz JR, Britt RD: **Cysteine as a ligand platform in the biosynthesis of the FeFe hydrogenase H cluster**. *Proc Natl Acad Sci U S A* 2015, **112**:11455-11460.

* This paper demonstrates the role of L-cysteine as a ligand platform to support HydG catalysed $Fe(CO)_2CN$ synthon assembly and release.

27. Suess DL, Kuchenreuther JM, De La Paz L, Swartz JR, Britt RD: **Biosynthesis of the [FeFe] Hydrogenase H Cluster: A Central Role for the Radical SAM Enzyme HydG**. *Inorg Chem* 2016, **55**:478-487.

* This paper includes a review of the key concepts involved in developing a mechanistic hypothesis for HydG.

28. Suess DL, Pham CC, Burstel I, Swartz JR, Cramer SP, Britt RD: **The Radical SAM Enzyme HydG Requires Cysteine and a Dangler Iron for Generating an Organometallic Precursor to the [FeFe]-Hydrogenase H-Cluster**. *J Am Chem Soc* 2016, **138**:1146-1149.

* This paper demonstrates the rather strict requirement for L-cysteine as the thiol ligand to achieve efficient turnover to form the HydG bound organometallic synthon.

29. Vallese F, Berto P, Ruzzene M, Cendron L, Sarno S, De Rosa E, Giacometti GM, Costantini P: **Biochemical analysis of the interactions between the proteins involved in the [FeFe]-hydrogenase maturation process**. *J Biol Chem* 2012, **287**:36544-36555.
30. Nicolet Y, Rubach JK, Posewitz MC, Amara P, Mathevon C, Atta M, Fontecave M, Fontecilla-Camps JC: **X-ray structure of the [FeFe]-hydrogenase maturase HydE from Thermotoga maritima**. *J Biol Chem* 2008, **283**:18861-18872.
31. Nicolet Y, Amara P, Mouesca JM, Fontecilla-Camps JC: **Unexpected electron transfer mechanism upon AdoMet cleavage in radical SAM proteins**. *Proc Natl Acad Sci U S A* 2009, **106**:14867-14871.
32. Rohac R, Amara P, Benjdia A, Martin L, Ruffie P, Favier A, Berteau O, Mouesca JM, Fontecilla-Camps JC, Nicolet Y: **Carbon-sulfur bond-forming reaction catalysed by the radical SAM enzyme HydE**. *Nat Chem* 2016, **8**:491-500.
33. Betz JN, Boswell NW, Fugate CJ, Holliday GL, Akiva E, Scott AG, Babbitt PC, Peters JW, Shepard EM, Broderick JB: **[FeFe]-hydrogenase maturation: insights into the role HydE plays in dithiomethylamine biosynthesis**. *Biochemistry* 2015, **54**:1807-1818.

34. Broderick JB, Byer AS, Duschene KS, Duffus BR, Betz JN, Shepard EM, Peters JW: **H-cluster assembly during maturation of the [FeFe]-hydrogenase**. *J Biol Inorg Chem* 2014, **19**:747-757.
35. Nicolet Y, Fontecilla-Camps JC: **Structure-function relationships in [FeFe]-hydrogenase active site maturation**. *J Biol Chem* 2012, **287**:13532-13540.
36. Adamska-Venkatesh A, Roy S, Siebel JF, Simmons TR, Fontecave M, Artero V, Reijerse E, Lubitz W: **Spectroscopic Characterization of the Bridging Amine in the Active Site of [FeFe] Hydrogenase Using Isotopologues of the H-Cluster**. *J Am Chem Soc* 2015, **137**:12744-12747.
37. Brazzolotto X, Rubach JK, Gaillard J, Gambarelli S, Atta M, Fontecave M: **The [Fe-Fe]-hydrogenase maturation protein HydF from Thermotoga maritima is a GTPase with an iron-sulfur cluster**. *J Biol Chem* 2006, **281**:769-774.
38. Berto P, Di Valentin M, Cendron L, Vallese F, Albertini M, Salvadori E, Giacometti GM, Carbonera D, Costantini P: **The [4Fe-4S]-cluster coordination of [FeFe]-hydrogenase maturation protein HydF as revealed by EPR and HYSCORE spectroscopies**. *Biochim Biophys Acta* 2012, **1817**:2149-2157.
39. McGlynn SE, Shepard EM, Winslow MA, Naumov AV, Duschene KS, Posewitz MC, Broderick WE, Broderick JB, Peters JW: **HydF as a scaffold protein in [FeFe] hydrogenase H-cluster biosynthesis**. *FEBS Lett* 2008, **582**:2183-2187.
40. Shepard EM, McGlynn SE, Bueling AL, Grady-Smith CS, George SJ, Winslow MA, Cramer SP, Peters JW, Broderick JB: **Synthesis of the 2Fe subcluster of the [FeFe]-hydrogenase H cluster on the HydF scaffold**. *Proc Natl Acad Sci U S A* 2010, **107**:10448-10453.
41. Czech I, Silakov A, Lubitz W, Happe T: **The [FeFe]-hydrogenase maturase HydF from Clostridium acetobutylicum contains a CO and CN- ligated iron cofactor**. *FEBS Lett* 2010, **584**:638-642.
42. Berggren G, Adamska A, Lambertz C, Simmons TR, Esselborn J, Atta M, Gambarelli S, Mouesca JM, Reijerse E, Lubitz W, et al.: **Biomimetic assembly and activation of [FeFe]-hydrogenases**. *Nature* 2013, **498**:66-69.
** This study reports an elegant and concise approach to hydrogenase activation, using a synthetic [2Fe] mimic and HydF to activate *apo*HydA to the vigorously active *holo*- form.
43. Esselborn J, Lambertz C, Adamska-Venkatesh A, Simmons T, Berggren G, Noth J, Siebel J, Hemschemeier A, Artero V, Reijerse E, et al.: **Spontaneous activation of [FeFe]-hydrogenases by an inorganic [2Fe] active site mimic**. *Nat Chem Biol* 2013, **9**:607-609.
* This study highlights a further short-cut to activate *apo*-hydrogenase by incorporation of the [2Fe] subcluster by direct insertion of a synthetic [2Fe] mimic.
44. Myers WK, Stich TA, Suess DL, Kuchenreuther JM, Swartz JR, Britt RD: **The cyanide ligands of [FeFe] hydrogenase: pulse EPR studies of (13)C and (15)N-labeled H-cluster**. *J Am Chem Soc* 2014, **136**:12237-12240.

Figure Legends.

Figure 1 (a) Assembly of the $[2Fe]_H$ cluster to activate an [FeFe]-hydrogenase (HydA). Labelled tyrosine derived atoms are shown in red and blue, nitrogen derived from another precursor is colored green [44]. **(b)** General H-atom abstraction mechanism by radical SAM enzymes. Ad, Adenine; Sub, second substrate; Met, methionine. **(c)** Formation of a HydG-bound organometallic synthon. FTIR stretching bands assigned to CO and cyanide are shown in red and blue respectively.

Figure 2. Structure and function of *Ti*HydG. **(a)** Structure of HydG showing the overall fold and cluster binding sites. The domains are colored as follows: N-terminal extension, pink; TIM barrel, green, C-terminal domain, blue. **(b)** HydG active site cavity. The α-helices (green) and β-sheets

(yellow) of the TIM barrel are shown for clarity. The surface of the tunnel linking the two cluster binding sites is shown in pale blue and includes the binding position of SAM. Tyrosine did not co-crystallize with *Ti*HydG, but modelling studies indicate the likely binding site (labelled Tyr). **(c)** *Ti*HydG auxiliary 5Fe-5S cluster. **(d)** The *Ti*HydG 4Fe-5S cluster. The labile iron is not present, leaving a 7.1 Å gap between the (hydro)sulfide and the imidazole of His265.

Figure 3. **(a)** A potential fragmentation mechanism for dehydroglycine to CO, cyanide and water. **(b)** Proposed role of cysteine in HydG catalysis. Adapted from reference [27].

Figure 4. **(a)** Structure of *Tm*HydE. Domains are colored as follows: N-terminal domain, pale blue; TIM barrel, red; C-terminal domain, yellow. The active site cavity surface is shown in grey and contains the SAM analogue, SAH (green) **(b)** Structure of *Thermotoga neapolitana* HydF dimer. Domains for each monomer are colored as follows: domain I, GTPase, red; domain II, dimerization, green; domain III, cluster binding, blue. The colors for the second monomer are paler. Potential cluster binding ligands are shown in yellow. **(c)** Hydrogenase activation using synthetic $[2Fe]_H$ mimics.

Figure 1

Click here to download high resolution image

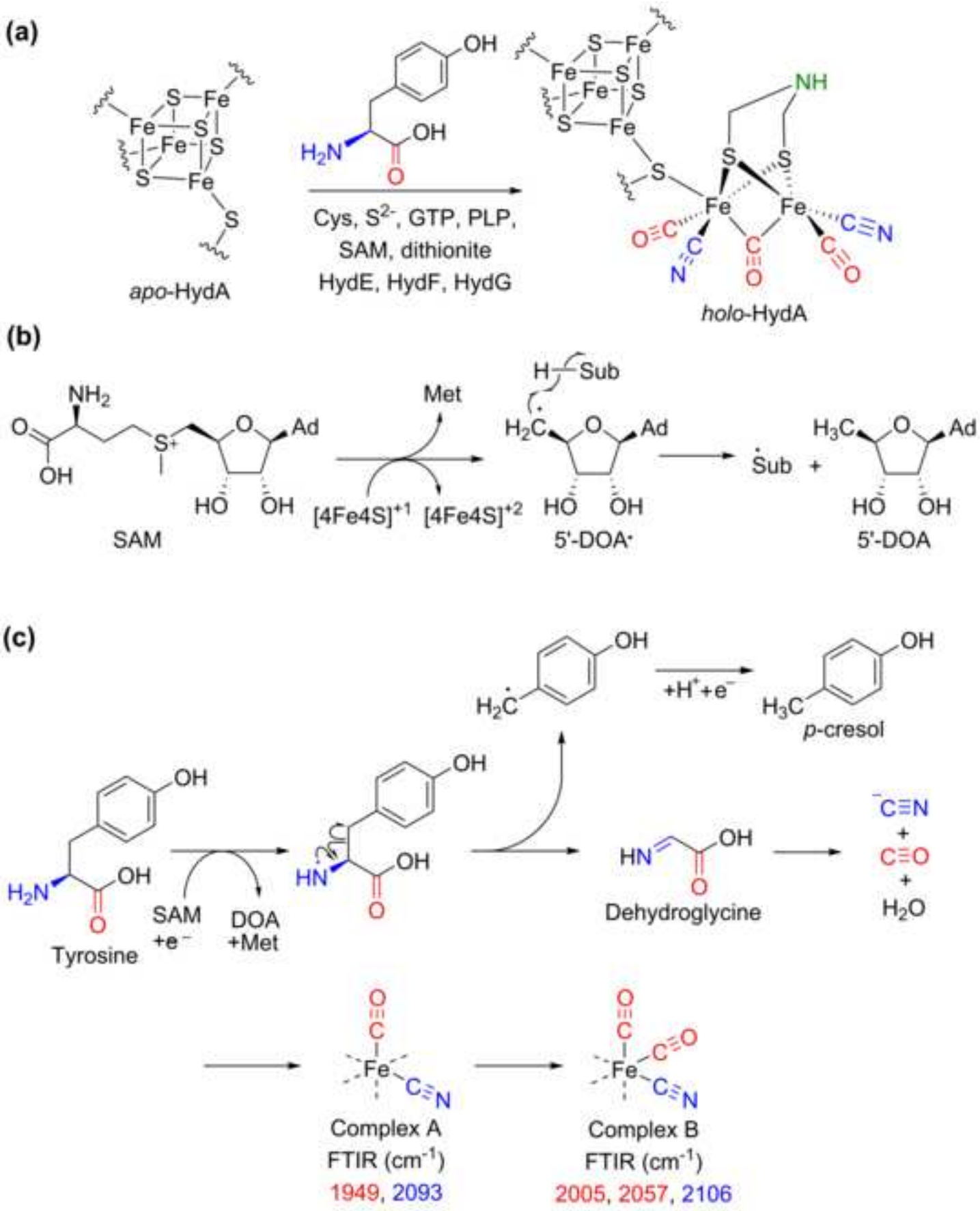

Figure 2

Click here to download high resolution image

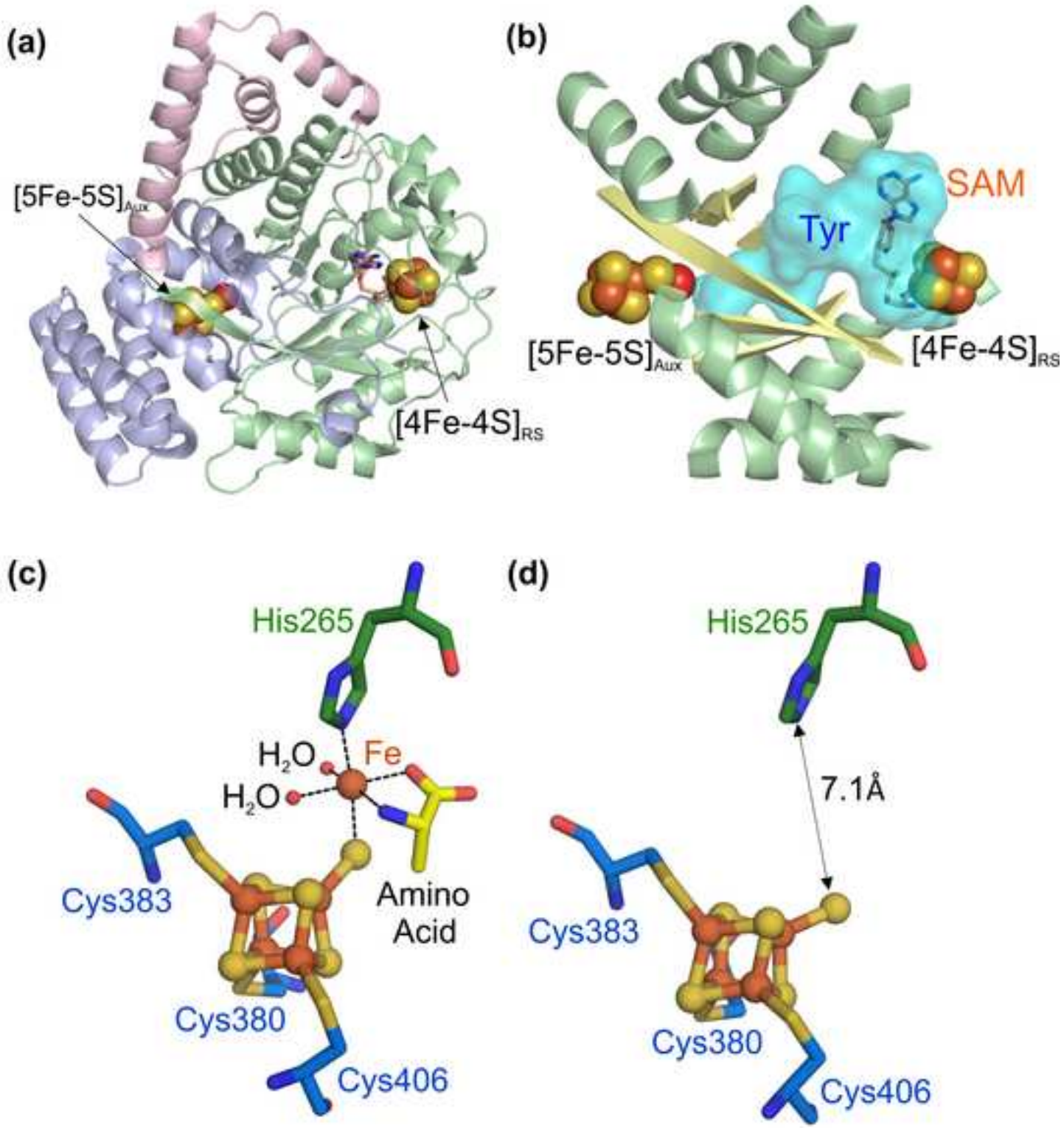

Figure 3

Click here to download high resolution image

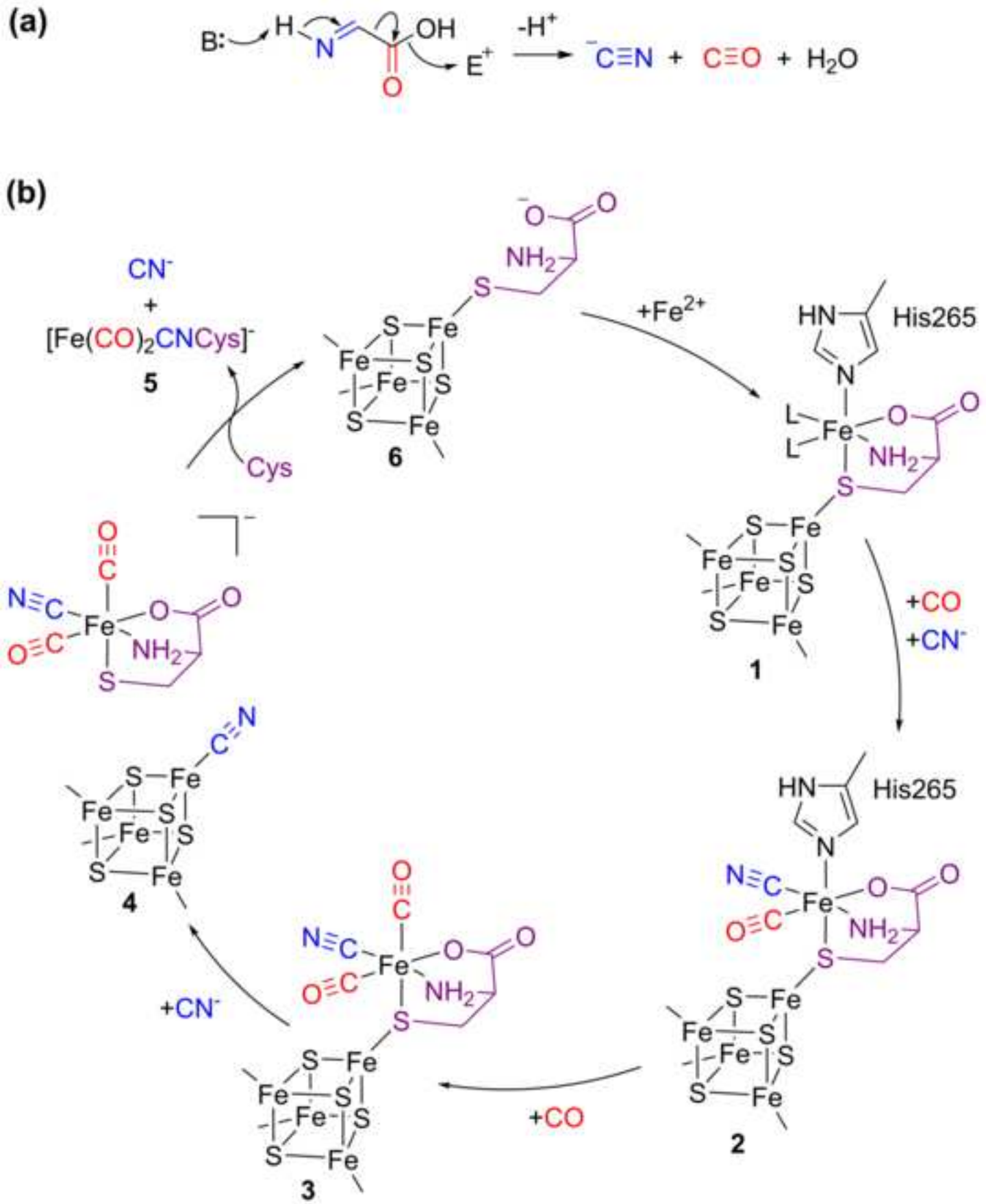

Figure 4

Click here to download high resolution image