A Radical Intermediate in Tyrosine Scission to the CO and CN^- Ligands of [FeFe] Hydrogenase

Jon M. Kuchenreuther[1†], William K. Myers[1†], Troy A. Stich[1], Simon J. George[1], Yaser NejatyJahromy[1], James R. Swartz[2,3], R. David Britt[1*]

[1]Department of Chemistry, University of California, Davis, Davis, CA 95616 USA
[2]Department of Chemical Engineering, Stanford University, Stanford, CA 94305 USA
[3]Department of Bioengineering, Stanford University, Stanford, CA 94305 USA

*To whom correspondence should be addressed; E-mail: rdbritt@ucdavis.edu.
†Equal contributors to this work.

The radical *S*-adenosylmethionine (SAM) enzyme HydG lyses free L-tyrosine to produce CO and CN^- for the assembly of the catalytic H-cluster of [FeFe] hydrogenase. We use electron paramagnetic resonance (EPR) spectroscopy to detect and characterize HydG reaction intermediates generated with a set of ^{2}H, ^{13}C, and ^{15}N nuclear spin labeled tyrosine substrates. We propose a detailed reaction mechanism in which the radical SAM reaction, initiated at an N-terminal [4Fe-4S] cluster, generates a free tyrosine radical bound to a C-terminal [4Fe-4S] cluster. Fragmentation of this tyrosine radical at the C_α-C_β bond forms a transient 4-oxidobenzyl (4OB$^\bullet$) radical, with the remaining dehydroglycine fragment bound to the C-terminal [4Fe-4S] cluster. Electron and proton transfer to this 4OB$^\bullet$ radical forms *p*-cresol with the conversion of this dehydroglycine ligand to Fe-bound CO and CN^-, a key intermediate in the assembly of the [2Fe] subunit of the H-cluster.

Microbial hydrogenase enzymes catalyze the redox interconversion of protons and H_2 using earth abundant metals in their catalytic centers, with [NiFe], [Fe], and [FeFe] classes known (*1*). The [FeFe] hydrogenases are adept H_2 producers, with turnover frequencies up to 10,000 s^{-1} (*2*). Their catalytic H-cluster (Fig. 1) consists of a conventional [4Fe-4S] cluster linked to a unique [2Fe] cluster that has two CN^- ligands, three CO ligands, and a dithiolate bridge with a central atom X, the chemical identity of which is ambiguous in current X-ray structures (*3, 4*). However, a nitrogen assignment to atom X is supported by recent reports of the assembly of active [FeFe] hydrogenase by incorporation of a synthetic [2Fe] subcluster with an azadithiolate bridge into apoenzyme (*5, 6*).

The HydE, HydF, and HydG maturases are Fe-S cluster-containing accessory proteins involved in the biological synthesis of the [2Fe] component of the H-cluster (*7*). HydE and HydG are members of the ever-growing family of radical SAM enzymes, characterized by a $CysX_3CysX_2Cys$ motif, where the three cysteine residues coordinate a [4Fe-4S] cluster through ligation of three Fe ions. The fourth Fe of the cluster binds *S*-adenosylmethionine (SAM) as an N/O chelate, and SAM is reductively cleaved to produce methionine plus a strongly oxidizing 5-deoxyadenosyl (5'-dA$^\bullet$) radical (*8, 9*). A diverse array of reactions are powered by the H-atom abstraction capability of the resultant 5'-dA$^\bullet$ radical, and current bioinformatics surveys reveal almost 50,000 members of the radical SAM enzyme class (*10*).

The focus of this report is the HydG maturase. This radical SAM enzyme generates CO, CN^-, and *p*-cresol using free tyrosine as its substrate (*11–14*). Although HydG has yet to be crystallographically characterized, biophysical data indicate that it has two [4Fe-4S] clusters (*15, 16*). The SAM-[4Fe-4S] cluster is bound near the N-terminus, whereas the second cluster is modeled as coordinated to three cysteine residues of a $CysX_2CysX_{22}Cys$ sequence near the C-terminus. Sequence homology with other radical SAM enzymes such as biotin synthase and the tyrosine lyase ThiH point to HydG having a triose phosphate isomerase (TIM) barrel structure,

in which eight α-helices surround a ring comprised of eight parallel β-strands that contain a buried active site suitable for small molecule substrates (*11, 17*).

Like HydG, ThiH also lyses tyrosine, in this case to generate dehydroglycine (DHG) as an intermediate in anaerobic thiamine biosynthesis (*18, 19*). ThiH lacks the second [4Fe-4S] binding domain of HydG. The EPR spectrum of the ThiH SAM-[4Fe-4S]$^{+}$ cluster is altered by SAM binding but unaffected by tyrosine incubation. In a proposed mechanism (*18, 19*), the initial 5'-dA$^{\bullet}$ radical abstracts the phenolic H of the tyrosine substrate, forming a neutral tyrosine radical, which is then cleaved at the C_{α}-C_{β} bond. Quantum chemistry calculations favored homolytic cleavage of this bond, to form a transient glycyl radical, given its lower energy pathway compared to heterolytic cleavage. Modeling the differences in reactivity between wild type HydG and a HydG mutant missing the C-terminal Fe-S cluster (HydG^{SxxS}), which produces some CN^{-} but no CO, led Nicolet and coworkers (*20*) to propose the same glycyl radical intermediate in the HydG mechanism, building on the thermodynamic argument for the mechanism in ThiH. However, no radical intermediates have been experimentally characterized for either enzyme.

Here, we report on the EPR spectroscopy of wild type *Shewanella oneidensis* HydG (HydG^{WT}) expressed in *Escherichia coli*. Such expressed HydG, combined with HydE and HydF, can be used for *in vitro* synthesis of the [2Fe] component of the H-cluster and concurrent activation of [FeFe] hydrogenase apoprotein (*14, 21*). The use of tyrosine isotopologs allows us to determine the identity and electronic structure of paramagnetic reaction intermediates, including a previously unrecognized radical species that derives from free tyrosine. Based on our results, we propose a mechanistic model for the formation of Fe-coordinated CO and CN^{-} ligands that are associated with the C-terminal Fe-S cluster of the HydG maturase, a key step in the assembly of the [2Fe] subunit of the H-cluster.

In order to explore the nature of free tyrosine binding in HydG, we compared both the low

field ($g>>2$) (Fig. 2A) and midfield ($g\approx2$) (Fig. 2B) regions of the *X*-band EPR spectra of sodium dithionite (DTH)-reduced HydG^{WT} mixed with either L-tyrosine (Tyr) or unreactive meta-L-tyrosine (m-Tyr), along with the spectrum of the HydG^{SxxS} mutant devoid of the C-terminal Fe-S cluster (*16, 20*). In the spectrum of reduced HydG^{WT} without tyrosine or SAM added, the $g\approx2$ signal (Fig. 2B*i*) can be well-simulated with a single *g*-matrix [2.045, 1.936, 1.908] (fig. S1a), and we assign it to the N-terminal [4Fe-4S]$^{+}$ cluster without SAM bound (*13, 15*). The low field region of the spectrum (Fig. 2A*i*) shows a broad signal, with maximum intensities at $g\approx9.5$ and $g\approx5$ that we assign to one or more high spin (HS) Fe-S cluster forms. The $g\approx2$ regions for the HydG^{SxxS} and m-Tyr substitutions show an identical signal, which can be simulated with a single *g*-matrix [2.007, 1.878, 1.839] (fig. S1b), and which we assign to the SAM-bound form of the N-terminal [4Fe-4S]$^{+}$ cluster (*13*). The HS EPR signal is unaltered in the m-Tyr sample, but completely absent in the mutant HydG^{SxxS}, indicating that it originates from the C-terminal cluster.

Addition of Tyr to reduced HydG^{WT} (Fig. 2A/B*iv*) results in a large decrease of the HS EPR signal (the remaining g=4.3 signal resembles that of adventitiously-bound Fe(III)), along with the addition of new $g\approx2$ region features, seen mostly as shoulders flanking the N-terminal cluster signal. The $g\approx2$ region of the spectrum is simulated with two distinct components, the unaltered N-terminal cluster signal of Fig. 2B*i* plus a new signal with a *g*-matrix [2.060, 1.910, 1.880] (fig. S1c). The loss of the HS EPR signal intensity and the addition of a new $g\approx2$ component indicates a spin state conversion triggered by tyrosine binding. [4Fe-4S] clusters bound by three cysteines may lose the unique Fe and convert to [3Fe-4S] forms, as initially discovered in the aconitase enzyme (*22*). One such [3Fe-4S] form, in "purple aconitase" (*23*) and a related model complex (*24*), show similar *g*-values (9.6 to 4.3) as we observe as the HS EPR signal of HydG before adding tyrosine. The spectral similarities suggest the possibility that the addition of tyrosine substrate to the HydG triggers the installation of the unique Fe site

in the C-terminal cluster, thereby producing the observed S=1/2 [4Fe-4S]$^{+}$ signal. Fig. 3 shows a possible model for tyrosine binding to the C-terminal cluster. In step I tyrosine binds as an N/O chelate to this fourth Fe, analogously to the methionine component of SAM when bound to the N-terminal cluster. This resting state enzyme model suggests a mechanism in which the C-terminal Fe-S cluster is directly involved in the enzyme activity, along the lines of certain other radical SAM enzymes such as biotin synthase where dethiobiotin is bound to a second Fe-S cluster as an intermediate in S-atom transfer (*25*).

In order to test for such a mechanism, we conducted freeze quench EPR experiments to trap and characterize paramagnetic intermediates during the HydG reaction. With the addition of DTH, SAM, and tyrosine to initiate the HydG^{WT} reaction (Fig. 2A/B*v*, 30 s timepoint), the HS C-terminal cluster signal remains small, and the $g\approx2$ region of the spectrum shows a complex set of peaks in the Fe-S spectral region, plus a new radical signal at g=2. The radical is transient, as measured in samples cryotrapped at varied timepoints in a set of rapid freeze quench (RFQ) experiments (Fig. 2C), and a maximum radical EPR signal is observed about 2 s after reaction initiation. Fig. 2D shows spectra for longer reaction times. The g=2 radical signal is greatly diminished at timepoints past 1 min, leaving a complex EPR lineshape arising from the two reduced [4Fe-4S]$^{+}$ clusters. Hyperfine sub-level correlation (HYSCORE) and electron nuclear double resonance (ENDOR) spectra (fig. S2) recorded at the prominent g=1.9 feature that forms during the HydG^{WT} reaction show coupled ^{15}N and ^{13}C features (with $^{13}C_9$,^{15}N-Tyr), suggesting that either tyrosine or a tyrosine-derived fragment is bound to the C-terminal [4Fe-4S] cluster during the reaction.

We determined the identity of the transient radical signal by using a number of tyrosine isotopologs as substrates in the HydG-catalyzed reaction. Fig. 4A shows a set of *Q*-band (34 GHz) EPR spectra, with the higher frequency chosen to give better resolution of the structured radical EPR signal. The EPR spectrum of the radical trapped in the natural abundance Tyr

sample (Fig. 4A*i*) is unaltered by ^{13}C-labeling at either the C_α (Fig. 4A*ii*) or the $\underline{C}OO^-$ carbon (not shown), proving that our cryotrapped radical is not the previously proposed glycyl radical intermediate (*20*). In contrast, the lineshape of the radical collapses with $^{2}H_7$,^{15}N-Tyr (Fig. 4A*iii*), a signature that has been used to identify tyrosine protein radicals (*26*). Neutral tyrosine (Tyr$^\bullet$) radicals, like those proposed to be produced by the initial phenolic H-atom abstraction in HydG (*20*), have been widely studied with advanced EPR and electronic structure calculations (*27,28*). Fig. 4B illustrates the DFT-derived spin density for such a Tyr$^\bullet$ radical. There is a large amount of spin density on the phenolic O as experimentally observed by ^{17}O labeling (*28, 29*), on C3 and C5 as observed by large hyperfine couplings to their respective protons, and on C1 which transfers large and generally inequivalent hyperfine couplings to the two C_β protons.

The remaining spectra of Fig. 4A show that we have, in fact, trapped a different radical during the HydG reaction. The spectrum of the 3,5-$^{2}H_2$-Tyr sample (Fig. 4A*iv*) is almost identical to that of the natural abundance Tyr sample (Fig. 4A*i*), whereas substituting two additional deuterons on C2 and C6 (Fig. 4A*v*) causes a dramatic change, showing that electron spin density has shifted from C3 and C5 to C2 and C6. The remaining resolved hyperfine couplings are to the two C_β protons, which show the 1:2:1 pattern diagnostic of two equivalently coupled protons. When these are instead deuterated (Fig. 4A*vi*), this 1:2:1 pattern is abolished, and we see the largest effect of all the labeled tyrosines, indicating that the greatest spin density is on C_β, with equivalent couplings to both bound protons. Moreover, there is no observed ^{17}O hyperfine coupling in the 4-^{17}O-Tyr sample (fig. S3), also inconsistent with a Tyr$^\bullet$ assignment. Instead, the pattern of hyperfine couplings, as analyzed through density functional theory (DFT) based simulations (fig. S3, table S1), points to a 4-oxidobenzyl (4OB$^\bullet$) radical (Fig. 4C) as the origin of the cryotrapped radical EPR signal. This 4OB$^\bullet$ radical, produced by tyrosine C_α-C_β fragmentation, has its highest spin density at the CH_2 terminus, which is sp^2-hybridized, giving large and equivalent couplings to these two C_β protons. The overall spin density pattern is

shifted by one atom relative to a parent Tyr$^{\bullet}$ radical, with higher spin density on C2, C4, and C6 than on C1, C3, and C5, and with a lowered spin density on O (Fig. 4C vs. 4B). The oxygen-protonated form of the 4OB$^{\bullet}$ (4-hydroxybenzyl radical) shows a similar calculated shift in its spin density pattern relative to a Tyr$^{\bullet}$ radical (fig. S3, table S1).

Tyrosine binding to the C-terminal cluster and the radical chemistry enabled by the 4OB$^{\bullet}$ radical are at the heart of our proposed mechanism (Fig. 3). Although the HydG^{SxxS} mutant does produce some CN^{-}, stopped flow FTIR spectroscopy shows that this occurs without concomitant generation of CO and on a hundred-fold slower timescale than the HydG^{WT} production of Fe-bound CO and CN^{-} (fig. S4). Thus the HydG^{SxxS}-derived CN^{-} is not enzymatically relevant, and instead occurs along some secondary path, such as oxidative decarboxylation of DHG (*30*) that which could be produced as in the ThiH enzyme that natively lacks this secondary [4Fe-4S] cluster.

Our proposed mechanism begins with SAM bound at the N-terminal cluster and tyrosine bound at the C-terminal cluster (Fig. 3 step I), with reductive cleavage of SAM generating the 5'-dA$^{\bullet}$ radical (Fig. 3 step II). This radical is quenched by the abstraction of a solvent exchangeable H-atom (see mass spectrometric data, fig. S5), consistent with the proposal (*20*) that 5'-dA$^{\bullet}$ abstracts the phenolic H of free tyrosine. The resulting Tyr$^{\bullet}$ radical is ligated to the C-terminal [4Fe-4S] cluster (Fig. 3 step III) and is not currently observed in the RFQ EPR experiments. The specific coordination geometry of the Tyr$^{\bullet}$ radical bound to the C-terminal [4Fe-4S] cluster may play a direct role in directing the subsequent C_{α}-C_{β} bond cleavage along the heterolytic pathway, concomitantly forming the observed 4OB$^{\bullet}$ radical and DHG ligated to the [4Fe-4S] cluster (Fig. 3 step IV). Free DHG is unstable, and rapidly hydrolyzes to produce glyoxylate and ammonia, as observed as byproducts in the ThiH reaction in the absence of the other required thiazole precusors (*19*). In the case of HydG, scission of the [4Fe-4S]-bound DHG occurs to yield Fe-bound CO and CN^{-} concomitant with electron and proton transfer

to the 4OB$^{\bullet}$ radical to form the *p*-cresol product of the HydG reaction (Fig. 3 step V). Given the facile interconversion between [4Fe-4S] and [3Fe-4S] forms observed in proteins such as aconitase (*22, 23, 31*) and ferredoxins (*32*), this unique CO- and CN^{-}- loaded Fe may then be inserted into the assembly of the [2Fe] subunit of the H-cluster. We are currently employing a variety of spectroscopy techniques to reveal further details of this fascinating metallocofactor assembly process.

References and Notes

1. P. M. Vignais, B. Billoud, *Chemical Reviews* **107**, 4206 (2007).

2. K. A. Vincent, A. Parkin, F. A. Armstrong, *Chemical Reviews* **107**, 4366 (2007).

3. J. W. Peters, W. N. Lanzilotta, B. J. Lemon, L. C. Seefeldt, *Science* **282**, 1853 (1998).

4. A. S. Pandey, T. V. Harris, L. J. Giles, J. W. Peters, R. K. Szilagyi, *Journal of the American Chemical Society* **130**, 4533 (2008).

5. G. Berggren, *et al.*, *Nature* **499**, 66 (2013).

6. J. Esselborn *et al., Nature Chemical Biology Advance Online Publication* (2013).

7. D. W. Mulder, *et al.*, *Structure* **19**, 1038 (2011).

8. P. A. Frey, A. D. Hegeman, F. J. Ruzicka, *Critical Reviews in Biochemistry and Molecular Biology* **43**, 63 (2008).

9. J. L. Vey, C. L. Drennan, *Chemical Reviews* **111**, 2487 (2011).

10. http://sfld.rbvi.ucsf.edu/django/superfamily/29/.

11. E. Pilet, *et al.*, *FEBS Letters* **583**, 506 (2009).

12. R. C. Driesener, *et al.*, *Angewandte Chemie-International Edition* **49**, 1687 (2010).

13. E. M. Shepard, *et al.*, *Journal of the American Chemical Society* **132**, 9247 (2010).

14. J. M. Kuchenreuther, S. J. George, C. S. Grady-Smith, S. P. Cramer, J. R. Swartz, *PLoS ONE* **6**, e20346 (2011).

15. J. K. Rubach, X. Brazzolotto, J. Gaillard, M. Fontecave, *Febs Letters* **579**, 5055 (2005).

16. C. Tron, *et al.*, *European Journal of Inorganic Chemistry* pp. 1121–1127 (2011).

17. Y. Nicolet, J. C. Fontecilla-Camps, *Journal of Biological Chemistry* **287**, 13532 (2012).

18. M. Kriek, *et al.*, *Journal of Biological Chemistry* **282**, 17413 (2007).

19. M. Kriek, F. Martins, M. R. Challand, A. Croft, P. L. Roach, *Angewandte Chemie-International Edition* **46**, 9223 (2007).

20. Y. Nicolet, L. Martin, C. Tron, J. C. Fontecilla-Camps, *Febs Letters* **584**, 4197 (2010).

21. J. M. Kuchenreuther, R. D. Britt, J. R. Swartz, *PLoS ONE* **7**, e45850 (2012).

22. H. Beinert, M. C. Kennedy, C. D. Stout, *Chemical Reviews* **96**, 2335 (1996).

23. M. C. Kennedy, *et al.*, *Journal of Biological Chemistry* **259**, 4463 (1984).

24. A. J. M. Richards, A. J. Thomson, R. H. Holm, K. S. Hagen, *Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy* **46**, 987 (1990).

25. C. J. Fugate, *et al.*, *Journal of the American Chemical Society* **134**, 9042 (2012).

26. B. A. Barry, G. T. Babcock, *Proceedings of the National Academy of Sciences of the United States of America* **84**, 7099 (1987).

27. M. L. Gilchrist, J. A. Ball, D. W. Randall, R. D. Britt, *Proceedings of the National Academy of Sciences of the United States of America* **92**, 9545 (1995).

28. R. J. Hulsebosch, *et al.*, *Journal of the American Chemical Society* **119**, 8685 (1997).

29. F. Dole, B. A. Diner, C. W. Hoganson, G. T. Babcock, R. D. Britt, *Journal of the American Chemical Society* **119**, 11540 (1997).

30. R. Michaels, L. V. Hankes, W. A. Corpe, *Archives of Biochemistry and Biophysics* **111**, 121 (1965).

31. T. A. Kent, *et al.*, *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **79**, 1096 (1982).

32. S. J. George, F. A. Armstrong, E. C. Hatchikian, A. J. Thomson, *Biochemical Journal* **264**, 275 (1989).

Acknowledgements:

This work was funded by the Division of Chemical Sciences, Geosciences, and Biosciences (RDB: award # DE-FG02-11ER16282) and the Division of Material Sciences and Engineering (JRS: award # DE-FG02-09ER46632) of the Office of Basic Energy Sciences of the United States Department of Energy. We thank Dr. Daniel Suess for useful discussions on the proposed HydG mechanism.

Fig. 1. The catalytic H-cluster of [FeFe] hydrogenases. Ball-and-stick representation (from Protein Data Bank entry 3CY8) was generated using UCSF Chimera: Fe (brown), S (yellow), C (gray), O (red), N (blue), unknown atom X (magenta), H not shown for simplicity.

Fig. 2. Continuous wave EPR spectra of DTH-reduced HydG samples. (A) Low field (9.37 GHz, 12 K except as noted, 5 mW) and (B) $g\approx2$ region (9.37 GHz, 20 K, 0.1 mW) X-band CW EPR spectra of (*i*) reduced HydG^{WT} without Tyr or SAM, (*ii*) reduced mutant HydG^{SxxS} plus Tyr and SAM (10 K), (*iii*) reduced HydG^{WT} plus m-Tyr and SAM, (*iv*) reduced HydG^{WT} plus Tyr, no SAM, and (*v*) reduced HydG ^{WT} plus Tyr and SAM. (C) Kinetics plot of the integrated Q-band radical EPR signal from HydG^{WT} reaction samples trapped by rapid freeze quench methods. The red line is a fit to a sequential [A]$\rightarrow$[B]$\rightarrow$[C] model. (D) X-band (9.37 GHz, 20 K, 0.1 mW) EPR spectra of hand-freeze quenched reduced HydG^{WT} mixed with Tyr and SAM at varied timepoints (min).

Fig. 3. A proposed mechanism for the HydG-catalyzed production of Fe-bound CO and CN^- ligands from free tyrosine.

Fig. 4. Continuous wave EPR spectra of the freeze-quenched HydG^{WT} reaction with specific Tyr isotopologs, along with corresponding DFT based electronic structures. (A) Q-band (34.17 GHz, 112 K, 9.6 μW) EPR spectra of 30 s reaction samples with specific Tyr isotopologs. (i) natural abundance Tyr, (ii) $^{13}C_\alpha$-Tyr, (iii) $^2H_7$,^{15}N-Tyr, (iv) 3,5-$^2H_2$-Tyr, (v) 3,4,5,6-$^2H_4$-Tyr, and (vi) β,β-$^2H_2$-Tyr. (B) DFT-derived spin density of a neutral tyrosine (Tyr$^\bullet$) radical. (C) DFT-derived spin density of the observed benzylomethyl-4-olate (BM4$^\bullet$) radical.